# FEEDING PHYSIOLOGY OF THE COLD WATER APPENDICULARIAN OIKOPLEURA VANHOEFFENI (TUNICATA)



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# FEEDING PHYSIOLOGY OF THE COLD WATER APPENDICULARIAN *OIKOPLEURA VANHOEFFENI* (TUNICATA)

by

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# Thesis abstract

Clearance and ingestion rates, behavior, gut passage time, pigment degradation and assimilation efficiency were examined for *Oikopleura vanhoeffeni* (Appendicularia, Tunicata) in a gradient of concentrations of laboratory grown diatoms. Data from particle removal experiments and behavioral observations showed similar trends, although the magnitude of the responses varied depending on the technique used. Clearance rate decreased significantly with food concentration, although saturation was not reached for a particle concentration range representative of conditions in the field. No lower feeding threshold was found. Clearance rate also decreased with the age of the external filtering device (i.e. house). For an individual animal, a wide range of clearance rates can be encountered from 0 to a physiological upper limit given by the Morris and Deibel (1993) model. Using body size, tail beat frequency and proportion of time spent feeding from *in situ* observations it was possible to estimate clearance rate in the field. The means of these estimated clearance rates were within a threefold range of the means of five alternative methods. The mean gut passage time of *O. vanhoeffeni* was 0.8 h and was independent of trunk length and particle concentration, although it varied significantly among individuals. Studies with $^{68}$Ge incorporated into the silica frustules of diatoms as a conservative tracer, showed that chlorophyll *a* conversion (i.e. degradation into fluorescent and non-fluorescent breakdown products) was on average 79%. The chl *a* conversion was not related to the amount of food in the gut estimated by visual inspection, although it was inversely correlated with the amount of $^{68}$Ge and chl *a* recovered in the guts of animals. Assimilation efficiency of bulk diatom carbon was 67%. Extraction in various solvents allowed fractionation of food and feces into four main biochemical pools. Proteins and low molecular weight compounds were preferentially

absorbed by the animals over lipids and polysaccharides. Predicted C:N ratios (by weight) for fecal pellets produced on a diatom diet ranged from 6.0 to 7.2, depending on the formula used and were consequently not much higher than the C:N ratios of the ingested diatoms (C:N = 5.2 - 6.3). The results of this thesis are relevant for the calculation of realistic population clearance rates as well as for the biochemical transformation of sinking material as appendicularians in general are major grazers in the world oceans.

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# General Introduction

The class Appendicularia is a dominant taxonomic group of zooplankton in the world's oceans. Appendicularians range from the tropical and temperate waters to the polar oceans, and from surface waters to the deep sea (Alldredge 1976, Fenaux 1993, Shiga 1993, Hopcroft and Roff 1995). Their complex water filtering device (referred to as the 'house') is capable of efficiently removing particles of a wide size range, from colloids to diatoms (Deibel 1986, Flood et al. 1992). Since appendicularians remove small particles from suspension and produce large particles such as fecal pellets and houses, they play a key role in the vertical flux of organic carbon, shunting carbon with a short residence time in the upper mixed layer to the deep sea where residence times are decades or longer (Fortier et al. 1994). Submicrometer particles usually considered to be too small to be utilized by copepods contribute a large portion of the ingested material in appendicularians (Bedo et al. 1993). Appendicularians graze on the entire range of organisms from bacteria to microzooplankton which are part of the so called 'microbial loop' (Urban 1992 et al.). This has important implications for modeling food webs. Like other pelagic tunicates, appendicularians seem to be capable of taking advantage of pulses of primary production, channeling material and energy efficiently into growth and reproduction (Alldredge and Madin 1982).

Despite the overall significance of appendicularians in marine pelagic systems, they have not been studied in as much detail as copepods, for example, partly due to difficulties in working experimentally with gelatinous zooplankton (Hamner et al. 1975). Conventional zooplankton net tows usually destroy a large portion of gelatinous zooplankton, which biases our view of the composition of the mesozooplankton community towards the more hardy Crustacea (Hamner et al. 1975).

The species of interest in my study is the abundant, cold-water appendicularian *Oikopleura vanhoeffeni* Lohmann. It is a relatively large appendicularian with a maximum house diameter of 5 cm, and is generally found in polar and subpolar waters (Shiga 1993), characteristically at water temperatures ranging from -1.5 to 5 °C. *O. vanhoeffeni* drifts with the cold Labrador Current from the Greenland shelf to the coast of Newfoundland, where it can be observed and collected in the surface waters by SCUBA divers from January to June. During the summer this species can still be found in deeper water (Deibel, unpubl.), although it disappears from the warmer surface layers where water temperatures reach 12 °C. *O. vanhoeffeni* is also very abundant in the polynyas of northeast Greenland and in the Bering Sea, although its occurrence in the North Pacific has not been described until recently (Shiga 1993, Acuña et al., in prep.).

In this thesis, I examine the three components of feeding of this species in response to food concentration: clearance rate (chapters 1 and 2), ingestion rate (chapters 1 and 3) and assimilation rate of organic nutrients (chapter 4). The first chapter covers the

functional feeding response using clearance rate, two selected behavioral variables (tail beat frequency and proportion of time spent feeding), fecal pellet volume and gut passage time as response variables to changing food concentrations. In the second chapter, I test the hypothesis whether clearance rate can be estimated from size and behavioral observations in the field. The third chapter examines the gut pigment technique as a possible method to measure ingestion rates in field. The fourth chapter focusses on the assimilation efficiency of bulk carbon of diatoms and preferential absorption of major biochemical fractions. In all manipulations, laboratory reared diatoms were used as the experimental food source since they represent suitable model particles. Diatoms display wide ranges in their abundances in Newfoundland waters with low values during the oligotrophic conditions of the summer months (Pomeroy et al. 1991), to peak abundances during the spring bloom (Navarro and Thompson 1994). Diatoms also contain indigestible silica frustules, which I will use as naturally occuring indigestible tracers (chapter 3 and 4). This study emphasises the distinction between clearance and ingestion rate, since in appendicularians the number of particles removed from suspension by the external filters is not equal to the number of particles ingested. The general findings are not restricted to *O. vanhoeffeni*, and will either be directly applicable to other appendicularians or aid in formulating new working hypotheses on similar species.

# Chapter 1. Functional feeding response and behavioral ecology of *Oikopleura vanhoeffeni*

## 1.1 Introduction

The functional response concept describes how prey mortality relates to prey abundance. The concept was originally introduced by Solomon (1949), and later Holling (1959a), not only to describe the feeding rates of organisms quantitatively, but also to understand and explain behavioral modifications in response to changing resource levels. In the latter sense, functional responses and observations of behavior are closely linked (Holling 1959b). In the more than 30 year history of functional response theory for copepods (Adams and Steele 1966), originally descriptive models (e.g. Mullin et al. 1975) have been expanded either to form optimal foraging theory (Lehman 1976) and ecosystem models (Steele 1974), or to explain distributional patterns of various copepod species (Paffenhöfer and Stearns 1988). However, a variety of organisms within the zooplankton community are different from copepods in their mechanisms of food collection (Alldredge and Madin 1982). Considering various feeding strategies and adaptations of a wide range of zooplankton will probably increase the variety of functional response models beyond those developed for copepods, perhaps leading to the understanding of principles and constraints common to all zooplankton rather than to one specific group.

Studies on the feeding of appendicularians have resulted in contradictory evidence about the functional response pattern. Paffenhöfer (1975) investigated feeding of an appendicularian (*Oikopleura dioica*) in the laboratory using a phytoplankton mixture (*Isochrysis galbana*, *Monochrysis lutheri* and *Thalassiosira pseudonana*). Logarithmic regression equations relating clearance rate to body size were calculated for two different

food concentrations, although the difference between the regressions was not statistically significant. Without presenting further data, however, Paffenhöfer (1975) stated that the clearance rate of *O. dioica* 'is a function of food concentration' and 'increases with decreasing food concentration'. From *in-situ* grazing experiments with *O. dioica* and *Stegasoma magnum*, Alldredge (1981) found decreased clearance rate for *S. magnum* at increased concentrations of particulate organic carbon. However, confounding factors such as temperature could not be ruled out and Alldredge (1981) concluded that 'controlled studies' were needed to determine the effects of food concentration on the grazing rates of that species. King (1981) determined optimal food concentrations of nanophytoplankton (*Isochrysis galbana*) to maximize growth and survival rates for *O. dioica*. Only animals maintained at food concentrations of 62.5 and 125 µg C/l attained maturity, but animals fed lower and higher food concentrations grew more slowly and did not survive for a complete generation. In the same study, King et al. (1980) found no change in clearance rate in response to food concentration. Landry et al. (1994), on the other hand, found saturated feeding in *Oikopleura* sp. using gut fluorescence as an indicator for ingestion rate. In an overview of pelagic tunicates, Alldredge and Madin (1982) stated that the feeding rates of salps, doliolids, and appendicularians are independent of food concentration, and contrasted them to pelagic crustaceans, which filter at higher rates when food concentrations are reduced (e.g. Frost 1972). Knoechel and Steel-Flynn (1989) found that an increase in biomass of both chain-forming diatoms and plankton smaller than 2 µm equivalent spherical diameter (ESD) had an inhibitory effect on the clearance rate of *O. vanhoeffeni*. However, the abundance of intermediate-sized particles ranging from 2 to 30 µm (ESD) did not affect the clearance rate (Knoechel and Steel-Flynn 1989). The authors suggested that their observations could be explained by clogging of the inlet filter by large particles, and the food concentrating filter by small

particles (Knoechel and Steel-Flynn 1989). Thus, the central question of how appendicularian feeding relates to food concentration warrants further investigation.

The present study addressed this question in controlled laboratory experiments by measuring clearance rate, defecation interval and feeding behavior. Measurements of clearance rate are very useful for understanding the interactions of a suspension feeder with its environment, especially when the mode of particle capture is non-selective. The interval between the production of individual fecal pellets was used to investigate whether gut passage time changes with food concentration in continuously feeding animals. Gut passage time has been shown to vary with food concentration in animals with a pronounced functional response such as copepods (e.g. Dagg and Walser 1987). It is therefore an important response variable to food concentration where ingestion rates are of concern or where gut fullness is used as an indicator to estimate ingestion rates of animals in the field (chapter 3). Finally, behavioral observations add information about responses of an animal to changes in its food environment and elucidate mechanisms which may help to explain the shape of the functional response (Holling 1959a).

## 1.2 Material and Methods

### Collection

Individual specimens of *O. vanhoeffeni* were collected by SCUBA divers from January to June in Logy Bay (site of the Ocean Sciences Centre, Memorial University of Newfoundland) during 1993, 1994 and 1995. Since *O. vanhoeffeni* is a cryophile, the field season starts in January and usually ends in June, after which animals occur primarily in

deeper and colder layers beyond the reach of SCUBA divers. Animals of similar sizes were collected to reduce allometric effects. As shown in other studies, average clearance rates increase with tunk length following exonential curves with exponents ranging from 1.58 to 1.79 (Knoechel and Steel-Flynn 1989, Deibel 1988, respectively). Immediately after collection of individuals of *O. vanhoeffeni*, the jars were taken to the laboratory and placed into a glass holding tank with flowing ambient sea water. The temperature of the holding tank during the experiments was maintained at 2 ± 1 °C during all three field seasons. All experiments were performed in the original collection jars by replacing the lid of the jar with a specially designed lid with an inflow and outflow tube (Fig. 1.1).

General remarks about *O. vanhoeffeni*

Despite the fragile nature of *O. vanhoeffeni* and the extreme difficulties involved with keeping individuals alive for only a few days in the laboratory, there are some advantages to working with this particular species. The houses serve as protective spheres so that the animals can be retained in relatively small jars without coming into direct contact with container walls (Fig. 1.1). Individual animals of this species also filter sufficient volume of water to obtain a good signal to noise ratio in static and flow through experiments. Thirdly, the animals are large enough that their behavior can be observed with rather simple optical equipment while maintaining a good depth of field. These factors allowed me to record rate processes, in combination with time budget analysis of individual animals. For clarity, the various structures of animal and house to which this paper refers are depicted schematically in Fig. 1.2.

Experimental food sources

Since this study was focused on food quantity, laboratory-grown diatoms were used to control food quality. Diatoms are a good prey model for functional response studies since they display wide ranges of biomass in nature, with peaks during the spring bloom (Huntley 1981). *Thalassiosira pseudonana*, with 4 µm ESD, has been used previously for studies with appendicularians (Paffenhöfer 1975, Gorsky 1980). The advantages of using this small diatom include high growth rates in culture at room temperature, and the low volumetric food concentrations which can be measured accurately due to high cell numbers. The second alga used in these experiments was the larger *Thalassiosira nordenskioldii* (ESD = 15 µm), which is abundant in the natural habitat of *O. vanhoeffeni* (McKenzie et al., in press). In order to compare clearance rates of *O. vanhoeffeni* feeding on the two diatoms, the latter must be retained with the same efficiency. A set of preliminary experiments verified that the retention efficiencies for the two algae were identical (data not shown), which is consistent with the findings of Deibel and Lee (1992) with respect to the retention efficiencies of the pharyngeal filter. For each functional response experiment only one of the two algae was used.

All food concentrations are expressed as carbon equivalents instead of cell numbers in order to provide a common basis for comparisons with other studies. The carbon concentrations of the cells were calculated according to Strathmann (1967). An individual *T. pseudonana* cell therefore was considered to contain 8 pg C, an individual *T. nordenskioldii* cell 110 pg C. Since neither diatom formed chains, both could easily pass through the inlet filter of the animal (pore size > 80µm for the size range of animals used

in this study; Deibel 1986). Thus clogging of the inlet filter was eliminated as a source of variability.

The food concentration ranges for the experiments were chosen to lie within natural limits. Particular attention was paid to the low food concentration range (ca. 0.8 - 10 μgC $l^{-1}$), which is not frequently incorporated into functional response studies. These low carbon values are rare in the neritic environment in which *O. vanhoeffeni* occurs (Navarro and Thompson 1995) and may be more representative for deep water habitats of the open ocean (Menzel and Ryther 1964, Wangersky 1976). However, these low concentrations were necessary in my experiments to ensure detection of lower feeding thresholds *sensu* Frost (1975). The food concentrations ranged from 1.5 to ca. 1500 μg C $l^{-1}$ for the static experiments, and from 0.8 to 500 μg C $l^{-1}$ for all behavioral observations. In the flow-through experiments I focused on the range from 1.8 to 100 μg C $l^{-1}$ in order to determine whether there is a gradual change in clearance rate with food concentration, even in the low and medium concentration ranges. In this study I will frequently refer to 'low', 'medium' and 'high' food concentration. I arbitrarily assigned the term 'low food concentration' to concentrations < 10 μg C $l^{-1}$. The food concentration from 10 to 100 μg C $l^{-1}$ was termed 'medium' and overlaps with the range of ingestible carbon (particles <70 μm) of 29 to 85 μg C $l^{-1}$ found by Deibel (1988) at the collection site in Logy Bay and with the surface values given by Wangersky (1976) for the Atlantic and Pacific oceans. 'High' food concentrations were those exceeding 100 μg C $l^{-1}$. Values as high as 800 μg C $l^{-1}$ are typically found during the peak of the spring diatom blooms off Newfoundland (Navarro and Thompson 1995).

## Static experiments

Using a peristaltic pump, the water in the jars was periodically replaced with a fresh suspension of diatoms. Removal by the animals was monitored by taking subsamples from the inflow tube (Fig. 1.1). Subsamples of 10 ml were taken at time intervals of 30 to 60 min over the course of each experiment. The cell concentration of each 10 ml sub-sample was measured 6 times in a Coulter Counter Multisizer II and the mean value plotted as a single point on the line relating cell concentration to time elapsed. The average coefficient of variation was 9 % for repeated measurements of the subsamples. The slope of this regression over time represented the grazing coefficient (g) which was multiplied by the water volume in the container (435 ml) to yield clearance rate (Frost 1972). Three types of experiments were performed: (1) a series with low food concentrations was carried out to test the stability of clearance rates over time, both within and between animals; (2) a sequence of low, medium and low concentrations and (3) a sequence of medium, high and medium concentrations was used for each animal. The purpose of such manipulation was to avoid interactions of time and food concentration.

## Flow-through experiments

Particle removal experiments in static systems are considered the classical approach to study the functional response (e.g. Frost 1972, Marin et al. 1986), but they have one major disadvantage: the variable being tested (i.e. food concentration) decreases exponentially with time as a result of the animal's feeding activity. It is therefore difficult

to determine the long term response of an animal to a given particle concentration. In addition, when used in a functional response plot against food concentration, the 'independent variable' (i.e. food concentration) changes in response to the feeding activity of the animal and is therefore not truly independent. This problem was addressed by Marin et al. (1986), who suggested the use of initial concentration instead of mean concentration for statistical analyses. Although this procedure removes some of the statistical bias, it does not solve the problem practically or conceptually: the initial food concentration is still only representative for a short period of time at the beginning of a feeding interval, while most of the time the animal is exposed to much lower concentrations. Finally, an animal with a lower clearance rate is exposed to a high food concentration for a much longer period of time.

A flow-through experimental set-up is an alternative to a static system. While particle removal is still used to determine clearance rate, the particle concentration can be maintained at relatively constant levels. Flow-through systems have been used extensively in bivalve physiology (e.g. Møhlenberg and Riisgård 1979), but are rarely used to determine zooplankton feeding rates (Frost 1972), perhaps because zooplankton usually cannot be exposed to the turbulent water flow required. I therefore designed an experimental device which would allow for sufficient turbulent mixing without disturbing the delicate organisms. Essentially, the same set-up was used as in the static experiment, except that the inflow tube was connected to a peristaltic pump during the entire incubation period (Fig. 1.1a). To determine clearance rate, I used the formula given by Hildreth and Crisp (1976):

(1) $$CR = FR*(C_i - C_o)/C_o,$$

where CR is the clearance rate (ml $h^{-1}$), $C_i$ and $C_o$ are the inflow and outflow concentrations of the particles (cells $ml^{-1}$), respectively, and FR (ml $h^{-1}$) is the flow rate of water through the system. $C_o$ must be used as the denominator of the formula, not $C_i$, since to use $C_i$, the flow rate would have to be high enough that essentially no recycling of water occurred in the chamber (Hildreth and Crisp 1976). With large robust filter feeders such as mussels or ascidians this would be no problem. The fragile nature of *O. vanhoeffeni* and the comparably low clearance rate, however, required the alternative approach: keeping the flow slow and the jars sufficiently mixed (Hildreth and Crisp 1976). This was accomplished by pumping air bubbles into a perforated tube (Fig. 1.1b).

A simple set-up was devised to test whether the water in the jar was sufficiently mixed (i.e. the mean concentration in the jar = $C_o$). A jar without an animal was initially filled with filtered sea water which was slowly replaced by inflowing water with a known concentration of *T. pseudonana*. Two time series measurements were performed, one using air bubbles to create mixing as shown in Fig. 1.1, and the other without mixing. If the water is perfectly mixed, the particle concentration, monitored in the outflow, should follow hyperbolic tangent saturation kinetics. Since the air bubbles, which created enough turbulence for mixing, were confined within the Plexiglas tube, they did not come into direct contact with the animals. Observations with the dye methylene blue showed that water entered the Plexiglas tube at the bottom through the netting and returned to the jar through the holes on the upper part of the tube in short pulses (see arrows in Fig. 1.1b). Preliminary experiments with *O. vanhoeffeni* indicated that the animals resumed normal filtering behavior within a few minutes of the introduction of air bubbles. In these tests, I found the animals to habituate to high levels of turbulence (ca. 150 bubbles per minute). Since the feeding experiments were performed at much lower turbulence levels (ca. 50

bubbles per minute), I suggest that the experimental set-up did not interfere with the normal feeding behavior of *O. vanhoeffeni*. Initial experiments were performed to compare the flow through set-up with the static system. For these experiments low particle concentrations were used to minimize clogging of the food concentrating filters (< 8 μg C $l^{-1}$ of *T. pseudonana*). In approximately half of the experiments, clearance rate was measured by first applying flow-through conditions. The inflow was then stopped and the clearance rate was determined by static experiments as previously described. In the remainder of the experiments, the order of flow-through and static measurements was reversed.

Behavior

The behavior of the animals was recorded by means of a black and white video camera equipped with a wide angle lens (Kern Switar f = 10mm). For 9 animals, the image was greatly improved by using a macro-telephoto lens (Kern Macro-Switar f = 75 mm) with extension tubes and a color video camera (Sanyo Color CCD, model VDC-2972) which allowed high resolution imaging so that food particles could be followed through the entire gastro-intestinal tract. For 25 animals, behavior was monitored continuously throughout each flow-through experiment. The video recordings were used primarily to determine the proportion of time spent feeding and the tail beat frequency in response to food concentration. A single measurement of time spent feeding consisted of five feeding intervals and five pauses. The tail beat frequency was determined by randomly selecting a time interval within a certain feeding period and determining the elapsed time for ca. 30 beats with a stop watch. If the feeding interval was shorter than 30 tail beats, all tail beats within the whole feeding interval were counted. A tail beat was

defined as a full beat from the beginning to the return to the same position of the tail, analogous to the definition of one wave length (Morris and Deibel 1993). For nine animals, the length and width of each fecal pellet was measured in lateral view at the time of egestion. The fecal pellet approximates a prolate spheroid (Urban et al. 1992), so that volume is given by

$$V = (l * w^2 * \pi)/6, \quad (2)$$

where V is the volume ($mm^3$), l and w the length and width of the fecal pellet (mm), respectively. The measurements (length and width of the pellets) were taken with calipers from a video screen calibrated with a micrometer immersed in the experimental chamber. To facilitate accurate measurements the animals were positioned laterally to the camera. However, some error due to an angled view was unavoidable and estimated absolute volumes should be treated with some caution. Since the position of each animal did not change during the experiments, I assume the volumes are consistent at least within individuals. At higher magnification, the resolution of the video image is sufficient to observe whether the pharyngeal filter was in place. Experiments were terminated either when the animals left their houses, or after 10 h of observation. Experiments were also terminated if the trunk of the animal began to move relative to the house, indicating that the trunk was no longer attached to the house and clearance rate was close to 0. After the experiments, the trunks of the animals (excluding the gonads) were measured to the nearest 100 μm (Deibel 1988).

## 1.3 Results

### Test of the flow-through system

Fig. 1.3 shows that when the water was mixed using air bubbles the outflow concentration closely follows the curve predicted by hyperbolic tangent saturation kinetics. When the experiment was repeated without mixing, the outflow concentration departed from the predicted saturation kinetics and resembled a step function (Fig. 1.3). I therefore concluded that the water in the jars was sufficiently mixed by air bubbles to permit the use of equation 1. The employment of both static and flow-through set-ups on the same animals within a short period of time showed that both methods give very similar results (Fig. 1.4). The slope of the regression line was not significantly different from one ($n = 14$, $F = 2.8$ , $p = 0.12$, Fig. 1.4).

### Functional response determined in the static experiments

Examples of the time series measurements with individual animals are shown in Fig. 1.5. At a low food concentration, clearance rates remained constant (i.e. the slopes remained the same) over long periods of time (Fig. 1.5a). Fig. 1.5b, however, shows an example in which clearance rates steadily decreased until the house was replaced with a new one, resulting in higher clearance rate. The difference between the slopes (and hence clearance rates) of the old (79 ml $h^{-1}$) and the new house (238 ml $h^{-1}$) was ca. 3-fold. At a medium food concentration, some of the animals showed a reduction in clearance rate (Fig. 1.5c), whereas others maintained constant clearance rates throughout (Fig. 1.5d).

Fig. 1.5c also shows that the reduction in clearance rate can be reversible when the animal is re-exposed to a low food concentration. Fig. 1.5e displays a typical example for the high food concentration trials at concentrations representative of spring phytoplankton blooms. The clearance rate is reduced at high food concentration (>100 μgC $l^{-1}$), remains low when the medium food concentration is reached, and eventually levels out to very low values (i.e. 29 ml $h^{-1}$, Fig. 1.5e). This reduction in clearance rate after a pulse of high particle concentration implies that recent feeding history affects clearance rate as discussed below. The result is a five-fold range of clearance rates for the same animal within 30 h (Fig. 1.5e).

The statistical analyses with clearance rate as the response variable for the static experiments are summarized in Table 1.1. Although initial food concentration had a significant effect on clearance rate, very little of the overall variance could be explained by this factor (7%, Table 1.1). Even in the most conservative test for significance of this factor (when entered into the model in the last position, type III sum-of-squares), its influence could not be removed (Table 1.1). Fig. 1.6a shows clearance rate plotted against food concentration alone. The outlier close to the x-axis derives from an animal which was fed at high concentration before its clearance rate was measured (see feeding history below). The logarithmic regression shown in Fig. 1.6a indicates that the clearance rate changed by about 10-fold (i.e. from 348 to 36 ml $h^{-1}$) over the 1000-fold range of food concentrations presented to the animals. Although I tried to use animals of similar trunk length to control for body size, I tested whether the remaining range of trunk lengths had a significant effect on clearance rate. Trunk length had no effect on clearance rate in the static experiments and could therefore be excluded from the model (Table 1.1).

In addition to food concentration, a variable termed 'recent feeding history' was examined. The purpose of this variable was to elucidate the impact of clogging of the food concentrating filter on clearance rate. Using history instead of elapsed time was advantageous in the static experiments since food concentration changed with time (i.e. elapsed time and food concentration were not independent). I classified feeding history as follows: feeding history was assigned '1' if the food concentration in the preceding feeding interval was low (<10 μg C $l^{-1}$), '2' when the food concentration was between 10 and 100 μg C $l^{-1}$, and '3' when the food concentration in the preceding interval was higher than 100 μg C $l^{-1}$. For the first feeding intervals (i.e. unknown feeding history) feeding history was set to '0' (Fig. 1.7). If clogging were indeed a factor, one would expect a lower clearance rate after a period at high food concentration. Therefore, 'history' was incorporated into the general linear model (GLM) in Table 1.1 and examined separately (Fig. 1.7). A GT2-test was used for testing the differences between any pair of means for the classes of feeding history (Hochberg 1974, cit. in Sokal and Rohlf 1981). Overall, feeding history had a significant effect on the clearance rate (Table 1.1), although as shown in Fig. 1.7, the recent feeding history influenced clearance rate only when the preceding food concentration exceeded 100 μg C $l^{-1}$ (i.e. 'high concentration', Fig. 1.7).

Functional response determined in the flow-through experiments

A GLM was constructed to test the influence of animal size (i.e. trunk length), elapsed time and food concentration (Table 1.2). Since in this case all independent variables are continuous, the GLM is equivalent to a multiple linear regression with the equation:

(3) $cr = 269\ (\pm 30) - 17\ (\pm 8)\ time - 1.3\ (\pm 0.5)\ conc,$

where 'cr' is clearance rate (ml $h^{-1}$), 'time' is elapsed time (h), and 'conc' the food concentration ($\mu gC\ l^{-1}$). Values in brackets are standard errors of the estimates (n = 45). After stepwise removal of insignificant factors (including all possible interaction terms), the components of the model were tested using both type I and type III sums of squares (Table 1.2). As in the static experiments, trunk length was not significantly correlated with clearance rate because of the narrow size range chosen (Table 1.2). Both elapsed time and food concentration were highly significant using type I and type III sums-of-squares (Table 1.2). The effect of food concentration on clearance rate was small, considering that clearance rate decreased by ca. 2-fold over a 100-fold increase in carbon concentrations (Fig. 1.6b).

Behavior

The observed tail beat frequencies ranged from 0.52 to 1.77 $s^{-1}$ with a mean of 0.97 $s^{-1}$. The proportion of time spent feeding ranged from 0.09 to a maximum of 0.96 (i.e. the animal was filtering almost all the time). Time spent feeding was affected by the elapsed time, but not by food concentration (Table 1.3). Tail beat frequency was significantly affected by trunk length, elapsed time, and food concentration (Table 1.3). The feeding effort of the animal was defined as the product of time spent feeding and tail beat frequency and can be used as a proxy for clearance rate (Morris and Deibel 1993, chapter 2 of this thesis). The feeding effort ranged from 0.09 $s^{-1}$ to 1.40 $s^{-1}$, which means that the quantity which most closely represents the filtering activity varied about 16-fold. Feeding effort (and therefore the expected clearance rate) was significantly influenced by trunk length, elapsed time and food concentration (Table 1.3) and when regressed

separately against food concentration, it decreased significantly with increasing food concentration (Fig. 1.6c). Trunk length explained about 30% of the observed variance in tail beat frequency, but did not significantly influence time spent feeding (Table 1.3). All components of behavior shown in Table 1.3 (feeding effort, time spent feeding, tail-beat frequency and number of pauses) significantly decreased with elapsed time. The frequency of pauses was negatively correlated with trunk length and elapsed time, but was not influenced by food concentration. Fig. 1.8 shows two examples to document the range of responses of the animals to a change in food concentration. One animal increased its activity after the food concentration was switched to a lower level (Fig. 1.8a), whereas the other animal maintained the low feeding levels reached during the high food concentration interval (Fig. 1.8b). In both animals, a delayed response to a change in food concentration was apparent.

Mean values for tail beat frequency and time spent feeding for individual animals were not significantly correlated (Fig. 1.9a), although for some individuals, time spent feeding was significantly correlated with tail beat frequency (Fig. 1.9b). This indicates that both time spent feeding and tail-beat frequency may change in response to changes in general activity levels. However, it does not mean that animals with a high tail-beat frequency are also spending more time feeding. There was a strong relationship between clearance rate measured with the flow-through set-up and time spent feeding which explained ca. 50% of the total variance (Fig. 1.10). Since the animals were of similar sizes, those which spent a large proportion of their time feeding would also filter the largest volume of water, however, a linear regression line either has a positive x-intercept or the relationship is curvilinear. In either case, clearance rate is overestimated when feeding effort is used as a proxy and the proportion of time spent feeding is small.

Visual observations of defecation processes

For nine animals, the time interval between egestion of individual fecal pellets was monitored over many hours. In animals 2, 3 and 4 (Fig. 1.11), the pharyngeal filter was not in place for some period of time. As a consequence, no fecal pellets were produced during this time, and the interval between fecal pellets increased. This means that the time interval between fecal pellet egestion was affected by whether or not the pharyngeal filter was in place. When the intervals resulting from inactive pharyngeal filters were incorporated into calculating average defecation intervals, the mean value, as well as the variance around the means, increased (Fig. 1.11). When these intervals were excluded from calculation of the mean, the values were in much closer agreement with other animals which continuously produced the pharyngeal filter (ca. 16 min). The defecation intervals were not affected by food concentration, but differences between individuals were highly significant (Table 1.4). Independence of defecation intervals with respect to ambient food concentration was also demonstrated for individual animals (Fig. 1.12).

Since gut passage time remains constant (chapter 3), the volume of the pellet is directly proportional to ingestion rate, provided that the amount of open space within the pellet does not change. Since diatom frustules are incompressible, and the tunicate gut possesses no means of grinding the food, this assumption is met for a uni-algal diet. Using fecal pellet volume as a proxy for ingestion rate, the two animals presented in Fig. 1.13 display very different functional responses. Figure 1.13a shows a saturation response, whereas in 1.13b the fecal pellet volume was directly proportional to the food concentration. Fig. 1.13 also depicts the time axis in the experiments. In the experiment

shown in Fig.13b, the food concentration was steadily increased, whereas in the experiment shown in Fig. 1.13a, the food concentration was switched from high to low after maintaining the animal for 4 h at high concentration. This shows that, the functional response can take very different forms depending on the experimental conditions.

## 1.4 Discussion

### Static and flow-through experiments

The static experiments indicated significant changes in clearance rate with food concentration (Table 1.1, Fig. 1.6a). However, the static experiments potentially exaggerate the effect of particle concentration, since animals with low clearance rate are exposed to high food concentration for a longer period of time. Since animals show reduced clearance rate at high food concentration, the two variables (i.e. time and food concentration) are not independent. The flow-through experiments avoided this problem by keeping the food concentration relatively constant over many hours. The flow-through experiments showed that even in the range from low to medium food concentration, there is a significant reduction in clearance rate with increasing food concentration and time (Table 1.2, Fig. 1.6b). The effects of feeding history (Table 1.1, Fig. 1.7) and elapsed time on clearance rate and behavior (Tables 1.2 and 1.3) support the hypothesis that the activity of the animals and the clearance rate are influenced by clogging of the surface of the food concentrating filter (see below). Only a small portion (ca. 20%) of the total variance was explained by the full models in Table 1.1 and 1.2. The remaining variance

was caused by factors not accounted for in my experiments and could be linked to differences in qualities of the houses and filters and to inter-individual differences.

Modulation of feeding behavior

The intra- and inter-individual variability for some components of feeding behavior was very high. For example, individual feeding intervals ranged from as short as 3 seconds to as long as 12 minutes between tail arrests. The frequency of pauses (i.e. collapses of the feeding filter) ranged from 5 to 129 $h^{-1}$. The proportion of time spent feeding ranged from 9 to 96%. In contrast, tail beat varied much less, ranging from 0.52 to 1.77 $s^{-1}$. Considering this small plasticity in tail beat frequency in comparison with other variables, it is interesting that tail beat frequency was most strongly affected by body size, food concentration and age of the house (Table 1.3). It seems that tail beat frequency is a variable sensitive enough to detect the remaining allometric effects in the relatively narrow size range of animals collected for this study (Table 1.3).

Figures 1.5, 1.8 and 1.13 give an impression of the wide range of possible responses of individuals exposed to various food concentrations. For instance, Fig. 1.8 shows two different responses to a decrease in food concentration. However, the food concentration was ca. 50% higher in Fig. 1.8b than in 1.8a and the house of the animal in Fig. 1.8b may have been clogged permanently. In contrast, most of the particles may have come off the filter in the low concentration interval (Fig. 1.8a), allowing the animal to filter at higher rate at lower food concentration. On the other hand, there is a considerable range of responses even under identical conditions. One animal decreased its clearance rate at medium food concentration (Fig. 1.5c), another showed no response to the high

food concentration pulse (Fig. 1.5d). This lack of consistent behavior can be interpreted as a lack of generality, but emphasizes that each animal has a different feeding history and may respond to changes in food concentration differently. The observed variability among individuals is apparently not restricted to appendicularians. For example, individuals of the copepod *Calanus finmarchicus* displayed a similarly wide variety of responses when exposed to different concentrations of the same diet ranging from filtered sea water to 4000 cells $ml^{-1}$ (Turner et al. 1993). Thus, one needs to measure sufficient numbers of animals in order to describe 'average' response patterns, and my results caution against generalizing behavioral observations from few individual observations.

Does the number of pauses increase at high food concentrations?

Whenever an animal arrests its tail beat, the food concentrating filter collapses and particles come off the filter surface. When the animal subsequently resumes beating its tail, these particles are flushed onto the pharyngeal filter in one pulse. Since the number of collapses of the food concentrating filter coincide with the number of pauses, the number of pauses can be used to determine the number of backwashes. If back-washing is an effective way of cleaning the filter, one may expect an increase in the frequency of pauses (collapses of the house) with time and concentration of food, as suggested by Deibel (1988). As the data in Table 1.3 show, there was no indication that the frequency of pauses changed with food concentration. Rather, the number of pauses decreased significantly with time, which is clearly inconsistent with the hypothesis of increased numbers of backwashes with increased clogging of the house.

Comparison of clearance rate estimated by three different approaches

For particles retained with 100% efficiency, feeding effort can be converted into clearance rate according to the formula (Morris and Deibel 1993):

(4) $$cr\ (ml\ h^{-1}) = feeding\ effort\ (sec^{-1}) * w\ (mm) * A\ (mm^2) * 3.6,$$

where 'cr' is the expected clearance rate, 'feeding effort' the product of proportion of time spent feeding and tail-beat frequency, 'w' the tail width and 'A' the crossectional area of the body of water which is pushed at each tail beat. The constant of 3.6 converts $mm^3$ into ml and s into h. Since both *T. pseudonana* and *T. nordenskioldii* are retained with 100% efficiency, an expected clearance rate could be calculated for each observation of feeding effort from Fig. 1.6c. The expected clearance rate was then regressed against food concentration and this regression compared with those obtained from the static and flow-through experiments (Fig. 1.6d). ANCOVA was used to test whether regression slopes were significantly different in pairwise comparisons (Table 1.5). The slopes are represented by the interaction terms between the three types of experiment (static, flow-through or behavioral observations) and food concentration. Since no flow-through data at high concentration range existed, I compared the slopes using a restricted range (i.e. < 100 $\mu g\ C\ l^{-1}$, Table 1.5). The slopes were not significantly different between static and flow-through experiments, and between flow-through and behavior experiments (Table 1.5). However, the slopes for static and behavior experiments were significantly different over the limited (< 100 $\mu g\ C\ l^{-1}$) and the full ranges. One reason for the convergence between flow-through and behavior may be that behavior was recorded simultaneously on many of the animals in the flow-through experiments. The clearance rates measured using the three

methods converge in the concentration range from 10 to 100 μg C $l^{-1}$. Since natural concentrations of ingestible carbon are typically between 10 and 100 μg C $l^{-1}$ (Deibel 1988), *in situ* behavior can be used to estimate clearance rate during non-bloom conditions as shown in chapter 2 of this thesis.

Consequences for estimating feeding rates

There is a significant decrease in clearance rate with increasing food concentration, even for food levels < 100 μgC $l^{-1}$. The effect seems small, however, considering that a 100-fold increase in food concentration is accompanied by an approximately 2-fold decrease in clearance rate as measured by the flow-through technique (Fig. 1.6b). This suggests that a given individual may clear less than half of the water volume per unit time during the spring bloom than during the remainder of the year. However, at concentrations as high as 1000 μg C $l^{-1}$ (which may occur during the peak of a diatom bloom), the same animal may remove as little as 15% of the cells taken at low concentrations as determined by the static experiments (Fig. 1.6a). It is conceivable that houses may become clogged so rapidly that house turnover increases to a level which is no longer sustainable by the nutritional gain from the filtered food particles. A negative balance between energy gain and expenditure is therefore a likely cause of *Oikopleura dioica* mortality in experiments in which the animals were fed at food concentrations exceeding 500 μg C $l^{-1}$ (King 1981).

Comparison with other functional response studies

Paffenhöfer (1975) did not find a significant effect of food concentration on the clearance rate of *O. dioica*. This is not surprising, since the difference between the two particle concentrations offered was only ca. 2-fold and the 'signal' would not be visible through the intra- and inter-individual variability. In an *in situ* study of the feeding rates of *O. dioica* and *Stegasoma magnum* conducted by Alldredge (1981), particle concentrations also varied by less than two-fold. Again, given the high variability in functional response of individuals, the noise may have masked the expected signal. However, for *Stegasoma magnum*, Alldredge found decreasing feeding rates with increasing POC concentrations, but could not exclude confounding factors such as temperature. King (1981) measured clearance rate of *O. dioica* in the laboratory over a wide range of food concentrations. He concluded that clearance rate was not affected by particle concentration. Since this is the opposite of my conclusions, the discrepancy needs further attention. King (1981) performed single endpoint static experiments with single *O. dioica* in 250 ml jars over 24 h. Using an average clearance rate of 20 ml $d^{-1}$ as measured by King (1981) and an initial food concentration of 100 µg C $l^{-1}$, the final concentration according to the Frost equation (1972) would be 92 µg C $l^{-1}$. Considering that King (1981) had to account for the growth rate of algae in the jars, and also the underlying variability when working with individuals, it is highly unlikely that the author would have found the subtle changes in clearance rate with food concentration reported in the present paper. In my study much steeper slopes in the static experiments (one large *O. vanhoeffeni* in 500 ml over many hours), slow flows in the flow-through experiments, and repeated time series measurements of behavior were used. This resulted in a minimum of

experimentally induced variability and allowed detection of the weak trend between particle concentration and clearance rate.

Knoechel and Steel-Flynn (1989) showed that particles smaller than 2 μm ESD and larger than 30 μm influenced clearance rate, while the concentration of medium-sized particles was not related to clearance rate. This was attributed to clogging of the inlet and the food concentrating filters by large and small particles, respectively. In contrast, my study has shown that even particles of 4 and 15μm, which would fall within Knoechel and Steel-Flynn's (1989) medium particle-size fraction, influenced clearance rate when presented at different concentrations. In agreement with Knoechel and Steel-Flynn (1989), however, clogging of the filter surface is a plausible explanation for the observed variability. Using the gut pigment technique, Landry et al. (1994) found evidence of saturation of gut fluorescence at food concentrations above 1.5 μg chl a $l^{-1}$ in *Oikopleura* spp. in the Santa Monica Basin (California). Assuming a carbon : chl *a* ratio of 60, this critical concentration is equivalent to only 90 μg C $l^{-1}$, a concentration at which *O. vanhoeffeni* does not show saturation, according to my data.

Decreasing clearance rate at increasing particle concentrations have been reported for salps (Andersen 1985), while other authors have not found any effects of food concentration (Harbison and McAlister 1979, Deibel 1982). Petersen and Riisgård (1992) found that ingestion rates were saturated when the ascidian *Ciona intestinalis* was fed concentrations of more than 6000 and 16000 cells $ml^{-1}$ for small and large individuals, respectively. In these experiments, the flagellate *Rhodomonas sp.* with an ESD of 6 μm was fed to *C. intestinalis*. Using the logarithmic regression provided by Strathmann (1967) for the conversion of cell volume to cell carbon for 'other phytoplankton', the

critical concentrations above convert to 126 and 336 µg C $l^{-1}$, respectively. Considering the benthic habitat of *C. intestinalis*, these levels are surprisingly low. At these levels, *O. vanhoeffeni* did not show saturation, although feeding levels were reduced (Fig. 1.6, Tables 1.1 - 1.3). For comparison, the copepod *Calanus pacificus* (Frost 1972) showed critical concentrations similar to those of the ascidians, ranging from 100 to ca. 300 µg C $l^{-1}$, depending on the type of alga.

The problem of fitting conventional functional response models

There are several possibilities to explain observed functional response patterns, ranging from simple mechanistic explanations such as handling time (Holling 1959b) to more complex concepts such as energy optimization and optimal foraging (Lehman 1976, Willows 1992). In the first instance, the functional response is driven by the simple constraint of handling time, whereas in the latter a behavioral 'program' optimizes food intake rate and energy expenditure. Although handling time *per se* cannot be considered as an explanation for the observed functional response, since appendicularians simultaneously collect and process food, I present a conceptually similar idea of a passive effect of particle load on the animal. In the case of appendicularians this seems to involve decreased efficiencies of the food concentrating filters at high particle concentration over time (see below).

Since clearance rate gradually decreased over the whole range of food concentration, a type II functional response with a gradually decelerating curve may be a better description than a type I functional response, which assumes the presence of a critical concentration (i.e. a cut-off point below which clearance rate is constant). A type

III response is also unlikely, because clearance rate does not decrease at very low food concentrations (i.e. no lower feeding threshold is observable). The lack of a lower feeding threshold is not surprising, since the tail beat serves additional functions besides food acquisition such as generating a water jet for propulsion and exchanging the water in the house for respiration. Although of all three types, a type II functional response may conceptually be closest to my data, I refrain from fitting a saturation function. First, saturation was reached in only a few animals (Fig. 1.13a) but not in others (Fig. 1.13b), and fitting a saturation function to all data would be inappropriate. Secondly, considering the peculiarity of a time dependent functional response and the proposed mechanisms which lead to the observed functional response (see below), Holling's (1959b) disc equation would be descriptive at best and the estimated parameters (i.e. handling time and attack rate) meaningless. Since no statistical model is assumption free, I chose the simplest, linear relationship between clearance rate and food concentration to describe general trends. The logarithmic representations in Fig. 1.6 are primarily for display and should not be considered actual logarithmic functional response models. In both cases (linear and logarithmic), the assumptions of parametric statistics, such as normality of the residuals, were not violated.

How can one explain the decreasing clearance rate and feeding effort at high particle concentration and with increasing age of the house?

There are two possible mechanisms which could lead to higher water resistance in the food concentrating filter. First, even in the sophisticated tangential flow design of the food concentrating filter (Flood 1991), particles stick to the filter mesh, thereby reducing

the area through which water can pass. In older houses these permanently trapped particles scatter the light and make the filters clearly visible. Secondly, the 100- to 1000-fold concentrations of particles in the food concentrating filter (Morris and Deibel 1993) may influence the viscosity of the suspension. For small colloidal particles which are highly concentrated by *O. vanhoeffeni's* food concentrating filter (Flood et al. 1992), the viscosity increases with their volume fraction in the suspension (Einstein 1906). Considering additional electroviscous effects (Van de Ven 1989), the increase in viscosity due to the high concentration of particles in the house may be significant. In summary, the combination of clearance rate measurements and behavioral observations suggests a very simple interpretation of the functional response: decreasing filtration rates are caused by mechanical effects on the filter surface rather than a sensory-motor (behavioral) feedback loop.

What makes animals leave their houses?

Since the appendicularian is suspended in a microenvironment inside its house, it cannot determine whether the reduced number of particles intercepting the pharyngeal filter is caused by a clogged food concentrating filter or by low ambient food concentrations. In the first case, leaving the house is beneficial; in the latter, it would be an unnecessary loss of material. Lohmann (1909) suggested that the animal leaves its house when the filters are clogged or during predator encounter. Frame by frame analysis of my video recordings revealed that before fast-escape swimming behavior was initiated, the attachments between trunk and house were broken. The sequence of events is important, since the animal does not 'decide' to leave the house but rather responds to a dislocation of the trunk by initiation of the fast swimming response. Since the fast

swimming behavior can be triggered by stimulating either the epithelium or the Langerhans receptors (Bone 1985), and the bristles of the Langerhans receptor protrude into the house (Flood 1991), it seems that the Langerhans receptor represents a sophisticated 'booby trap' mechanism. Animals which fail to initiate the rapid tail beat become entangled in the mucus of the house and die. It is conceivable that the connections between house and animal wear out with time or break when the resistance to water flow due to clogging of the food concentrating filters increases. Fenaux (1985), however, found that in *Oikopleura dioica* house renewal is very regular over time even in nonfeeding animals (Fenaux 1985). However, the time interval between houses may be shortened if the rate of clogging is high. This is consistent with the variable number of uninflated rudiments found attached to the trunk in field collected *Oikopleura vanhoeffeni* (unpublished observation). Unfortunately, the house renewal rate was too low in our experiments to give reliable estimates for *O. vanhoeffeni.*

Independence of defecation interval from food concentration

For continuously feeding animals, the rates of defecation and therefore the gut passage times were not influenced by food concentration (Table 1.4, Fig. 1.12). As a result, fecal pellet volume changed markedly in response to changes in food concentration as shown in Fig. 1.13. However, the defecation interval varied significantly among individuals (Fig. 1.11). Since almost no mixing occurs, the guts of appendicularians (and probably the guts of other pelagic tunicates) are good examples of plug flow reactors as defined by Penry and Jumars (1987). Monitoring defecation intervals of individual animals in a gradient of food concentrations is probably the best assurance that no other factors influence the result, and that gut passage times remain constant over a wide range

of food concentrations (Fig. 1.12). For continuously feeding animals, the gut passage time equals the amount of food in the gut (in fecal pellet equivalents) times the average defecation interval. For continuously feeding individuals of *O. vanhoeffeni,* the amount of food equivalent to ca. 3 fecal pellets is present in the gut. The average defecation interval is 16 min, so the gut passage time should be 48 min (i.e. 3 x 16 min). This is precisely the average gut passage time of *O. vanhoeffeni* measured with diatoms and corn starch reported in chapter 3.

**1.5 Conclusions**

Food concentration and elapsed time significantly influenced the clearance rate of *O. vanhoeffeni.* Both clearance rate and behavioral components show similar trends, although the magnitude of the response varies depending on the technique. Conclusions about the nature of the functional response based on the observation of individual animals have to be treated with caution, since the functional response can take various forms depending on the experimental design and the feeding history of the animals. However, lack of stereotypical behavior when animals are exposed to varying food concentrations does not imply lack of generality. Overall, the various methods give consistent results: 1) Clearance rate decreases with increasing food concentration. 2) Clearance rate decreases with time. 3) Saturation is generally not reached even at the highest particle concentration. 4) No lower feeding threshold exists (i.e. clearance rate does not decrease at very low food concentration). 5) Gut passage time does not change with food concentration.

Owing to its unusual characteristics, the functional response of *O. vanhoeffeni* cannot be fitted into the classic functional response concept introduced by Holling

(1959a). However, since clearance rate decreases gradually with increasing food concentration, and a lower feeding threshold cannot be observed at low food concentrations, the obtained functional response resembles a type II response. The observed patterns are consistent with a simple hypothesis which considers clogging of the feeding structures as the principal reason for the reduced filtration rates. However, some of the effects of clogging of the filter are reversible when the animals are exposed to low particle concentrations. This suggests either that particles are dislodged from the feeding filter or that there are viscosity effects at high particle concentrations which prevent the animals from filtering at high rates when the particle concentrations in the houses are high. In the light of these observations, appendicularians seem to have a very reduced behavioral repertoire and seem to have solved problems of food acquisition by evolving sophisticated feeding structures rather than by plasticity in their behavior.

## 1.6 Tables

Table 1.1. Summary of the effect of trunk length, recent feeding history and food concentration on clearance rate as determined in static experiments. The original general linear model was written as:

$$cr_t = \beta_0 + \beta_1 * tl + \beta_2 * history + \beta_3 * conc + ... \text{ (interactions) } ... + \varepsilon_t$$

where $cr_t$ is the clearance rate at time t, history = proxy for recent feeding history (history = 0 when animals were in their first feeding interval, history = 1 when preceding feeding interval was conducted at a concentration < 10 $\mu gC\ l^{-1}$, history = 2 when preceding food concentration was between 10 and 100 $\mu gC\ l^{-1}$, history = 3 when preceding food concentration exceeded 100 $\mu gC\ l^{-1}$). 'conc' = food concentration in $\mu gC\ l^{-1}$. '$\varepsilon_t$' = residual error. Various interaction terms and trunk length were removed stepwise when not significant at the $\alpha = 0.05$ level. The p-values are based on type I and type III sum-of-squares.

dependent variable: cr ($ml\ h^{-1}$)

| | | type I | | | type III | |
|---|---|---|---|---|---|---|
| explanatory variables | df | $r^2$ | p | | p | |
| history | 3 | 0.079 | 0.0073 | | 0.017 | |
| conc | 1 | 0.072 | 0.001 | | 0.001 | |
| full model | 5 | 0.151 | 0.0002 | - | - | - |
| n observations = 139 | | | | | | |

Table 1.2. Summary of statistical analysis of the effect of trunk length, elapsed time, and food concentration on clearance rate determined in flow-through experiments. The original general linear model was written as:

$$cr_t = \beta_0 + \beta_1 * tl + \beta_2 * time + \beta_3 * conc + \ldots \text{(interactions)} \ldots + \varepsilon_t$$

where $cr_t$ is the clearance rate at time t, 'tl' = trunk length, 'time' = elapsed time in hours and 'conc' = food concentration in $\mu gC\ l^{-1}$.'interactions' indicates all interaction terms which have been removed stepwise when not significant at the $\alpha = 0.05$ level. $\varepsilon_t$ = residual error. p-values are shown for both type I and type III sums of squares.

dependent variable: cr ($ml\ h^{-1}$)

| | | type I | | type III |
|---|---|---|---|---|
| explanatory variables | df | $r^2$ | p | p |
| time | 1 | 0.11 | 0.0158 | 0.0294 |
| conc | 1 | 0.13 | 0.0092 | 0.0092 |
| full model | 2 | 0.24 | 0.0026 | - |
| n observations = 45 | | | | |

Table 1.3. Summary of the effect of trunk length (tl), elapsed time and food concentration on feeding effort (effort), tail beat frequency (tbf), time spent feeding (tsf) and number of pauses per hour (equivalent to the number of collapses of the food concentrating filter). The general linear model was written as:

$$y_t = \beta_0 + \beta_1 * tl + \beta_2 * time + \beta_3 * conc + \ldots \text{(interactions)} \ldots + \varepsilon_t$$

where $y_t$ (dependent variable) is either feeding effort, tail beat frequency, time spent feeding or frequency of pauses, tl = trunk length (mm), time = elapsed time (h), conc = food concentration ($\mu gC\ l^{-1}$). 'tl*time'was the only interaction term which was significant at the $\alpha$=0.05 level. All other possible interaction terms have been tested and have been removed stepwise when not significant. The p-values are based on type I sums-of-squares. n.s. = not significant at $\alpha$=0.05.

| explanatory variables | df | effort ($sec^{-1}$) | | tbf ($sec^{-1}$) | | tsf (dimensionless) | | pause frequency (n pauses $h^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|
| | | $r^2$ | p | $r^2$ | p | $r^2$ | p | $r^2$ | p |
| tl | 1 | 0.11 | < 0.0001 | 0.29 | < 0.0001 | - | n.s. | 0.18 | <0.0001 |
| time | 1 | 0.09 | < 0.0001 | 0.04 | 0.0004 | 0.05 | 0.002 | 0.03 | 0.014 |
| conc | 1 | 0.04 | 0.0014 | 0.08 | < 0.0001 | - | n.s. | - | n.s. |
| tl*time | 1 | n.s. | n.s. | 0.01 | 0.038 | - | n.s. | 0.03 | 0.011 |
| full model | - | 0.24 | < 0.0001 | 0.42 | < 0.0001 | 0.05 | 0.002 | 0.24 | <0.0001 |
| number of observations | | 193 | | 193 | | 198 | | 198 | |

Table 1.4. 1-factor ANCOVA of the effect of food concentration on defecation interval taking individual variability ('individual') into account.

dependent variable: defecation interval

| | | type I | | type III |
|---|---|---|---|---|
| explanatory variables | df | $r^2$ | p | p |
| individual | 4 | 0.156 | < 0.0001 | <0.0001 |
| conc | 1 | 0.001 | 0.567 | 0.119 |
| individual*conc | - | 0.026 | 0.158 | 0.158 |
| full model | 5 | 0.183 | < 0.0001 | - |
| total | 139 | | | |

Table 1.5. One-factor ANCOVA to determine significant differences between slopes of the different types of experiments (Fig. 1.6) at two food concentration ranges.

| | < 100 µg C $l^{-1}$ | | full range | |
|---|---|---|---|---|
| experiment | n | p | n | p |
| static ↔ flow-through | 135 | 0.134 | - | - |
| static ↔ behavior | 291 | 0.042 | 331 | < 0.0001 |
| flow-through ↔ behavior | 182 | 0.085 | - | - |

## 1.7 Figures

Fig.1.1a: Experimental set-up to measure particle removal in static and flow-through experiments. The peristaltic pump delivered diatom suspensions from the header tank either in pulses (static) or continuously (flow-through). In the static experiments, up to 8 replicates could be used simultaneously. b) The samples were either taken from the inflow pipe with a syringe (static) or from the outflow spout (flow-through). Air bubbles kept the suspension sufficiently mixed so that the outflow concentration was identical with the concentration surrounding the animal. The house of the animal was held in place by means of a wire frame (not shown). Minimum mortalities were achieved by using the original collection jars as the experimental vessels. The jars held 500 ml and measured ca. 10 cm in diameter.

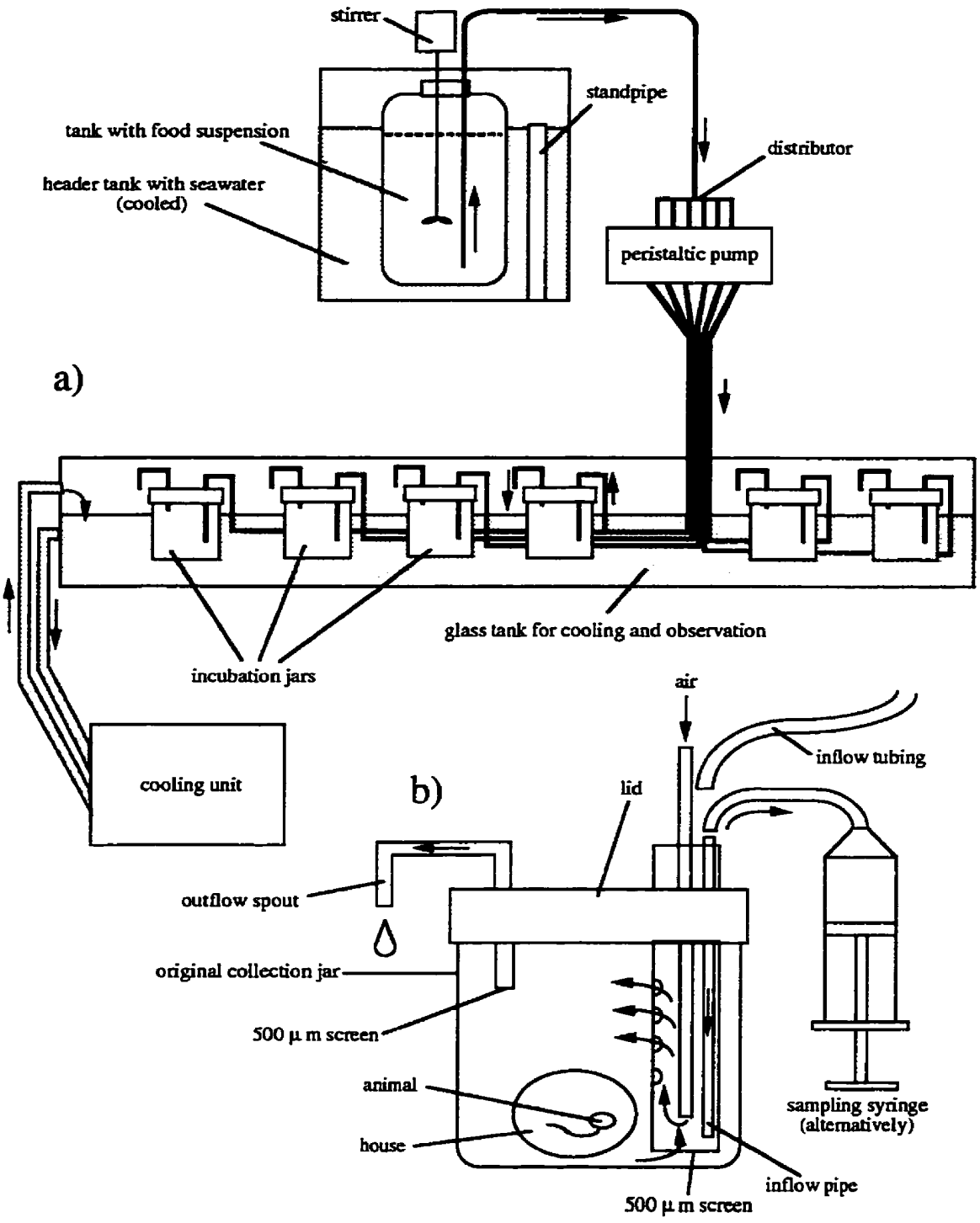

stirrer
standpipe
tank with food suspension
distributor
header tank with seawater
(cooled)
peristaltic pump
a)
glass tank for cooling and observation
incubation jars
cooling unit
air
inflow tubing
b)
lid
outflow spout
original collection jar
500 µ m screen
animal
house
sampling syringe
(alternatively)
inflow pipe
500 µ m screen

Fig.1.2: *Oikopleura vanhoeffeni* inside its house. Arrows indicate the flow of particles. When the animal is beating its tail, water enters through the paired inlet filters (only one filter shown) and passes through the tail chamber and the food concentrating filter (only one wing shown). The highly concentrated particle suspension then flows through the buccal tube into the pharynx, where it is caught on the pharyngeal filter. The filter, with the attached particles, is then swallowed by the oesophagus.

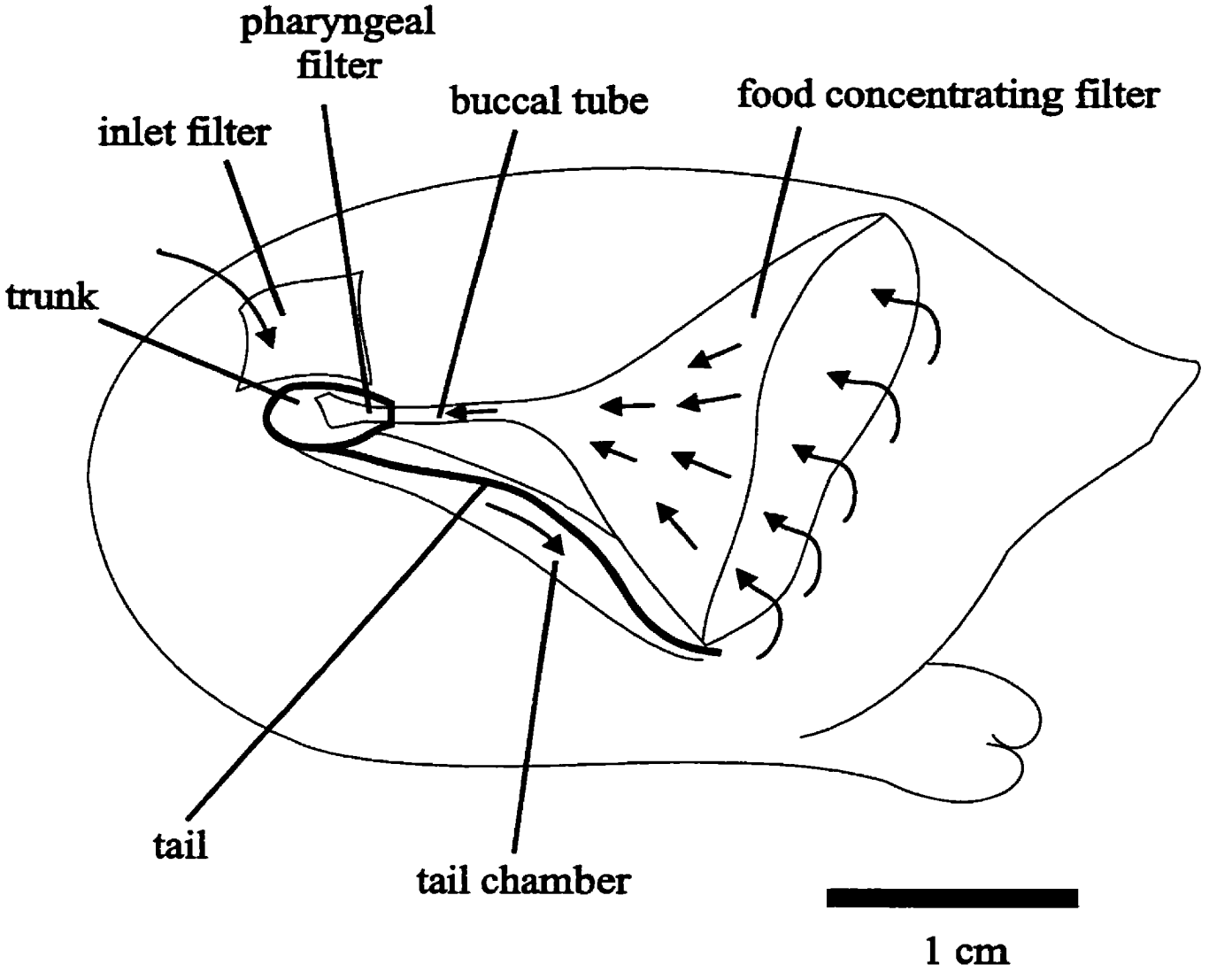

pharyngeal filter
buccal tube
food concentrating filter
inlet filter
trunk
tail
tail chamber
1 cm

Fig. 1.3: Experimental set up to test whether the suspensions in the jars were sufficiently mixed in order to satisfy the assumptions of equation 1 (see text). The jar was initially filled with filtered seawater, which was slowly replaced with a diatom suspension delivered by means of a peristaltic pump, and the outflow concentration was monitored over time. If sufficiently mixed, the particle suspension should follow a hyperbolic tangent function with the equation

$$C_o = (C_i * fr * t) / (V + fr * t),$$

where '$C_o$' and '$C_i$' are the particle concentrations in the outflowing and inflowing water (cells $ml^{-1}$), respectively, 'fr' the flow rate (ml $min^{-1}$), 't' the elapsed time from the start of water flow (min) and 'V' the volume of the jar (ml). The outflow concentration closely followed the predicted line in the mixed regime. However, when the experiment was repeated without bubbling (i.e. little mixing), the ouflow concentration resembled a step function over time. The bars are standard deviations of replicate counts with the Coulter Counter Multisizer.

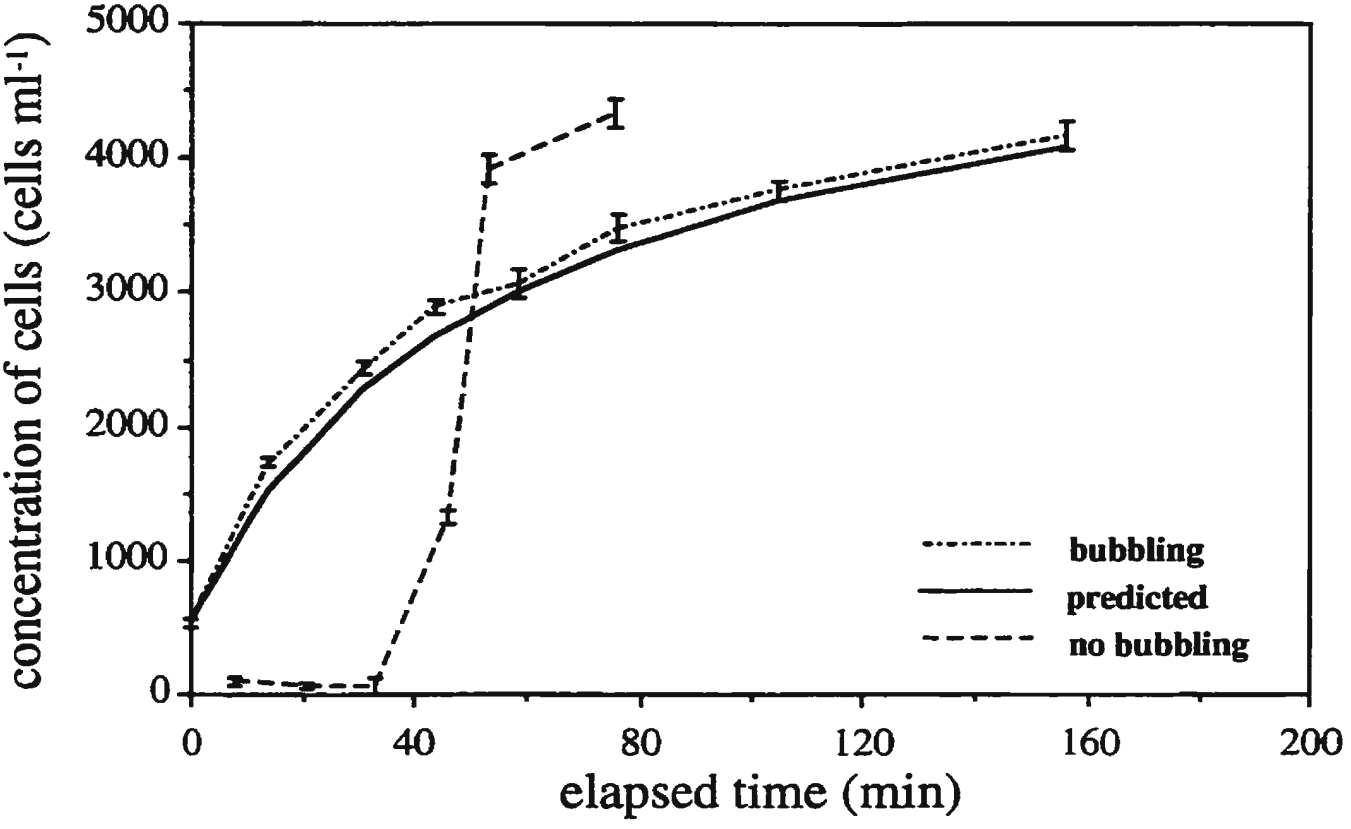

concentration of cells (cells ml-1)
5000
4000
3000
2000
1000
0
0
40
80
120
160
200
elapsed time (min)
bubbling
predicted
no bubbling

Fig. 1.4: Comparison of clearance rates determined from static and flow-through experiments. Clearance rates were measured on the same individuals using both set ups within a short period of time, using low food concentrations to avoid potential effects of clogging of the filters. The slope of the regression (solid line) was not significantly different from one (dashed line). Note: For this statistical analysis, the differences between x and y values were plotted against x and the resulting slope tested for significant deviation from 0.

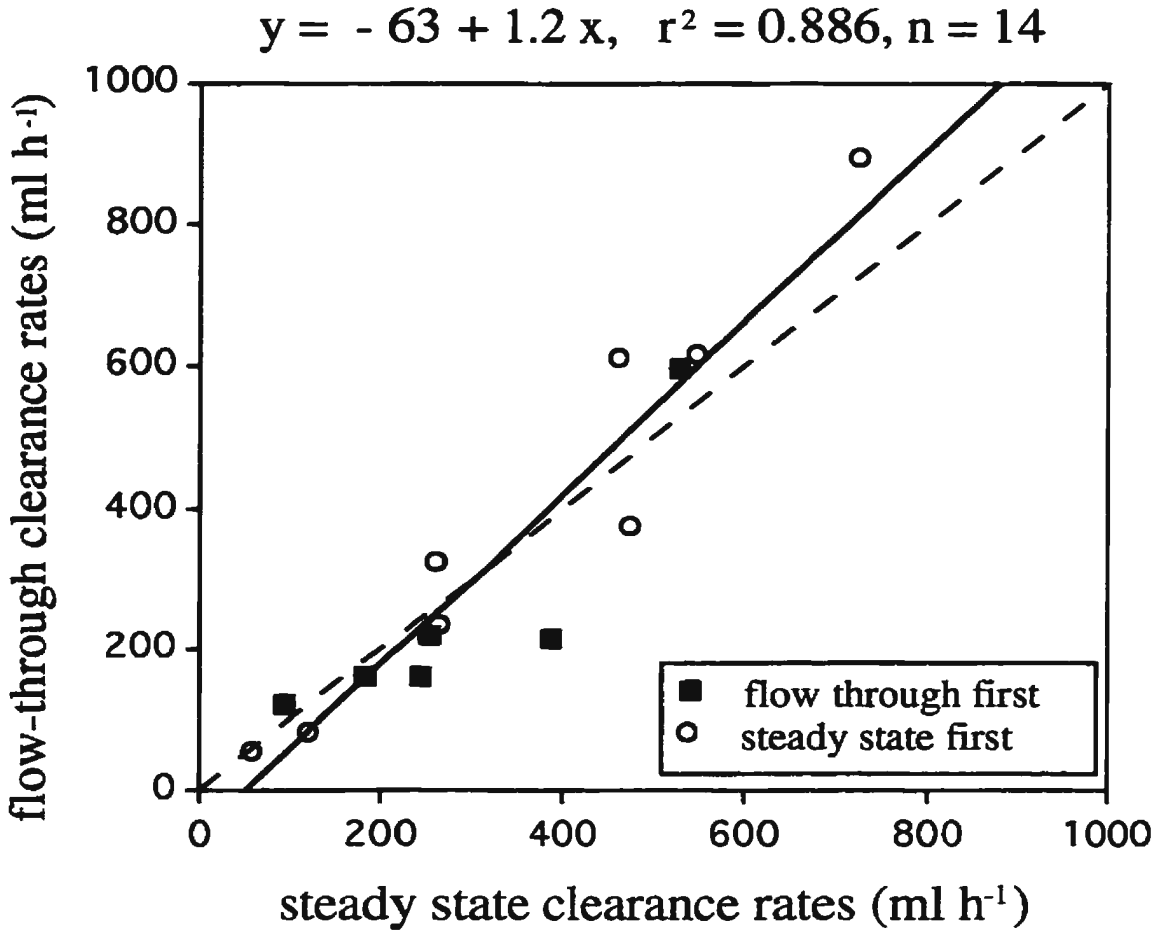

y = - 63 + 1.2 x, r² = 0.886, n = 14
flow-through clearance rates (ml h-1)
steady state clearance rates (ml h-1)
0
200
400
600
800
1000
flow through first
steady state first

Fig. 1.5. Examples of three different types of static experiments. a & b: repeated low concentrations; c & d low and medium concentrations; e: medium high and medium concentrations. The arrows indicate when the particle suspensions in the jars were renewed. Dashed lines indicate the arbitrarily chosen limits between the three levels of food concentration (low, medium and high). The numbers under the slopes are the corresponding clearance rates in ml $h^{-1}$. Fig. 1.5 a shows how constant the clearance rates were for some animals in the low food concentration trials. However, in some animals clearance rates decreased until a new house was built (1.5b). The difference between clearance rate in the old and the new houses was ca. 3-fold in this case. 1.5c shows an animal which displayed a significant reduction of its clearance rate when exposed to a medium food concentration. When the food concentration returned to a low value, the clearance rate recovered. Fig. 1.5 d shows an example of an animal which was not inhibited at exactly the same food concentration. Fig. 1.5e gives a typical example of a high food concentration trial. After a pulse of food at high concentration, the clearance rate of the animal did not recover, even after a medium particle concentration was attained.

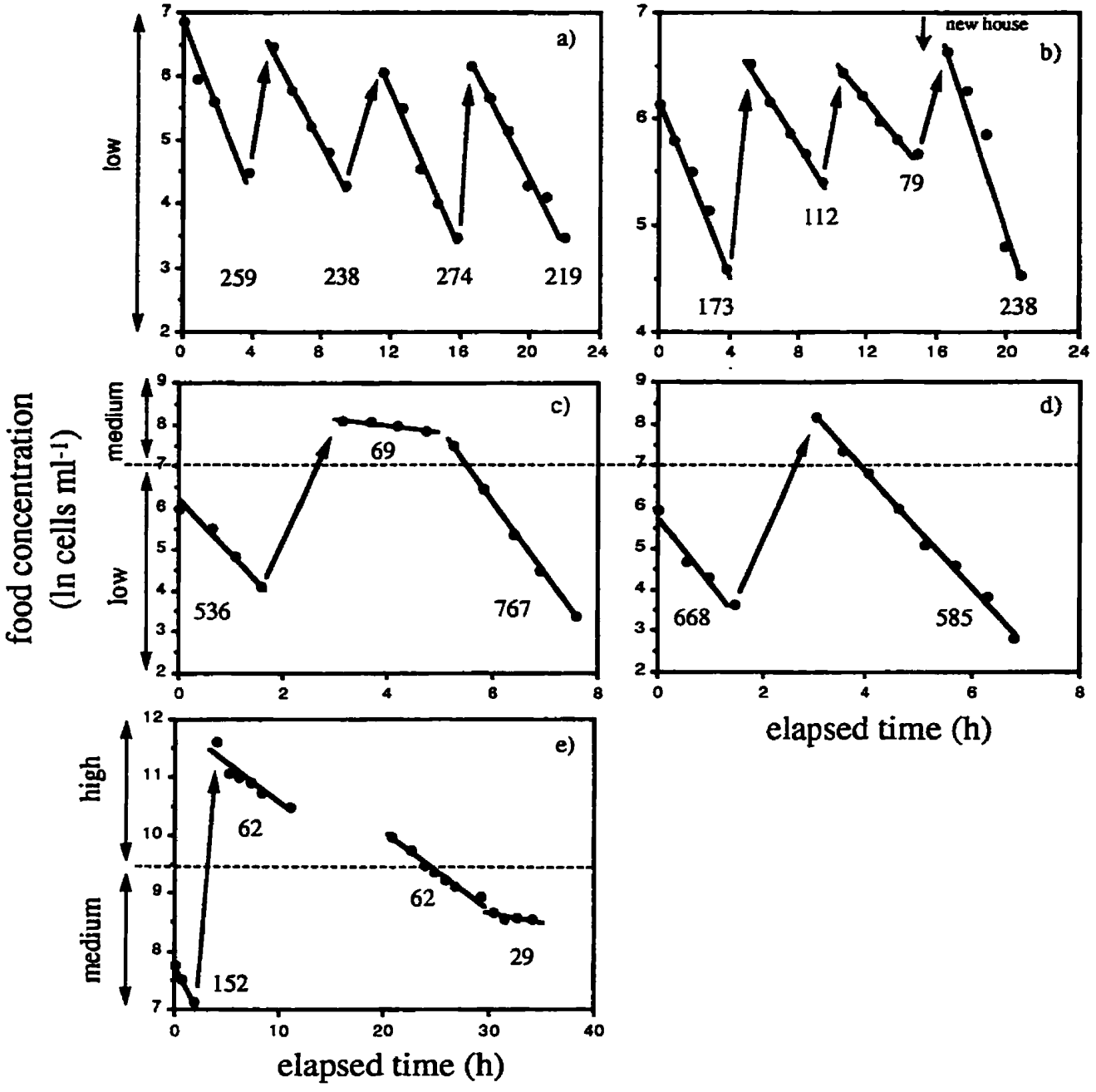

a)
b)
new house
c)
d)
e)
low
medium
high
259
238
274
219
173
112
79
238
536
69
767
668
585
62
62
29
152
food concentration
(ln cells ml-1)
elapsed time (h)
elapsed time (h)

Fig. 1.6: Log-log plots of the functional feeding responses of *Oikopleura vanhoeffeni* using 3 different techniques: a) static experiments, b) flow-through experiments and c) behavioral observations. Feeding effort is the product of tail beat frequency and time spent feeding. All three methods gave similar trends, although the magnitudes of the responses were different. For d) the behavioral observations have been converted into clearance rates using the Morris and Deibel (1993) model (dotted line) and compared to the regression lines for static (solid line) and flow-through (dashed line) experiments. The fine lines indicate the 95% confidence intervals for the three regression lines. Clearance rate measured in the static experiments showed the strongest response to food concentration, clearance rate estimated from behavioral observations the weakest.

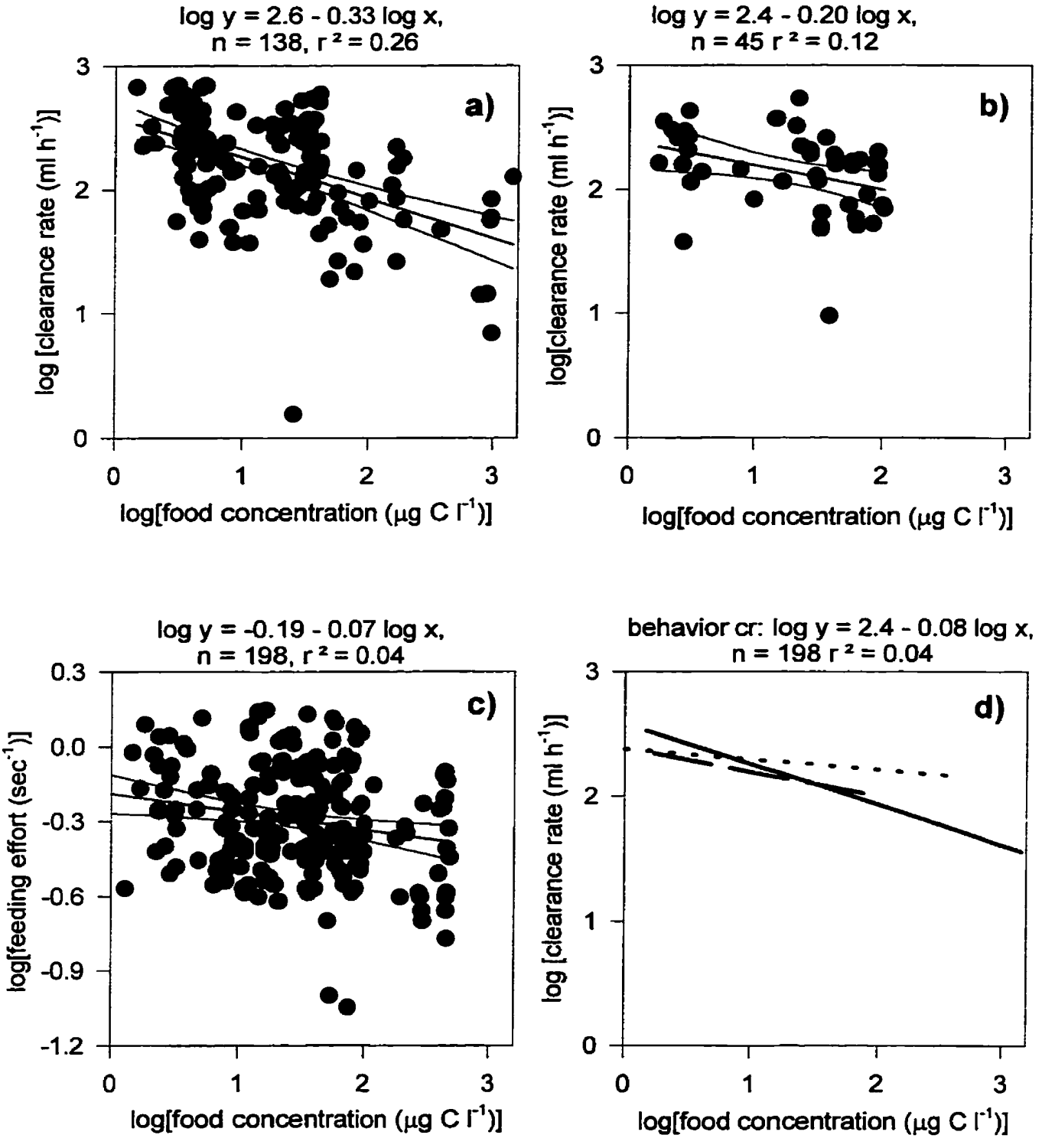

log y = 2.6 - 0.33 log x,
n = 138, r² = 0.26
a)
log [clearance rate (ml h⁻¹)]
log[food concentration (µg C l⁻¹)]
log y = 2.4 - 0.20 log x,
n = 45 r² = 0.12
b)
log[clearance rate (ml h⁻¹)]
log[food concentration (µg C l⁻¹)]
log y = -0.19 - 0.07 log x,
n = 198, r² = 0.04
c)
log[feeding effort (sec⁻¹)]
log[food concentration (µg C l⁻¹)]
behavior cr: log y = 2.4 - 0.08 log x,
n = 198 r² = 0.04
d)
log [clearance rate (ml h⁻¹)]
log[food concentration (µg C l⁻¹)]

Fig. 1.7: Effect of recent feeding history on clearance rate determined in static experiments. Feeding history was defined as in Table 1.1. Same letters indicate that there was no significant difference between means according to a GT2 test for pairwise comparisons (Sokal and Rohlf 1981).

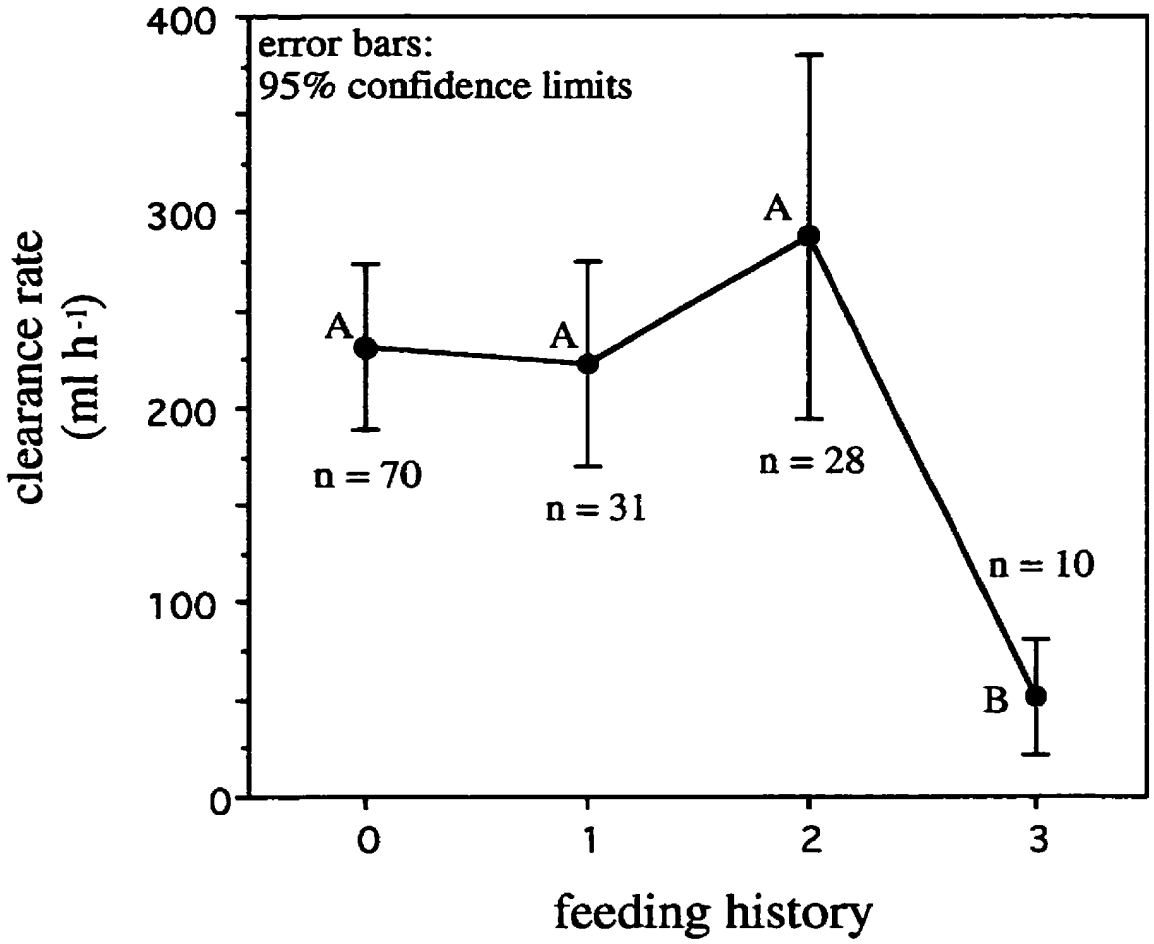

400
300
200
100
0
error bars:
95% confidence limits
clearance rate
(ml h-1)
A
A
A
B
n = 70
n = 31
n = 28
n = 10
0
1
2
3
feeding history

Fig. 1.8: Example of the behavior (i.e. feeding effort) of two individual *O. vanhoeffeni*, recorded on video, in response to switching the food concentration from high to low. In one case, the animal increased its activity after the switch in food concentration (a), in the other case feeding effort remained low. Note however that the initial high food concentration in (a) was about 50% higher than that in (b).

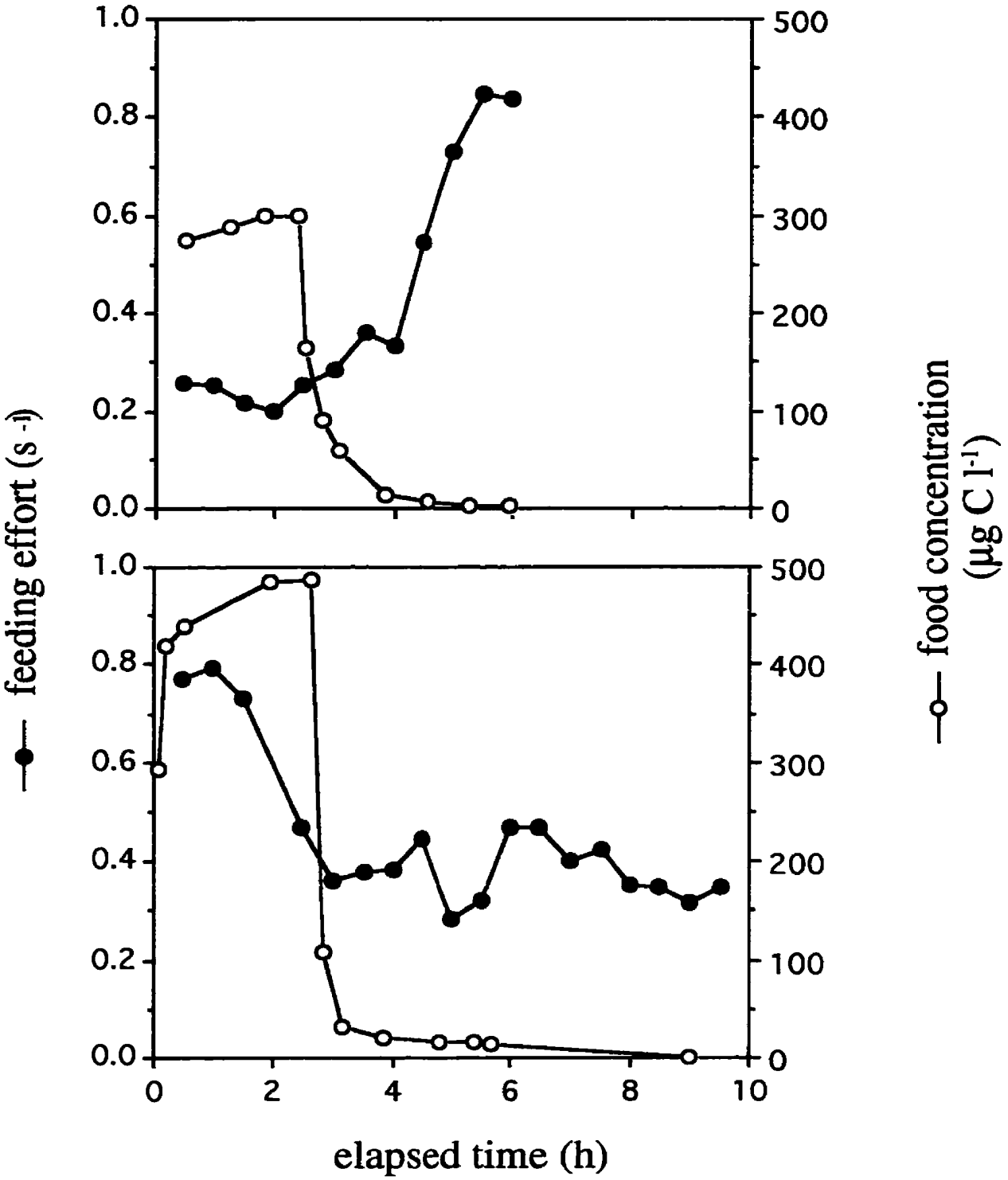

1.0
0.8
0.6
0.4
0.2
0.0
500
400
300
200
100
0
0
2
4
6
8
10
elapsed time (h)
feeding effort (s -1)
food concentration (µg C l-1)

Fig. 1.9: Comparison of tail beat frequency and time spent feeding when the data were pooled for individual animals (a) or when the two variables were plotted for a single individual (b). This shows that while both variables may still be connected by a general activity level within one individual, animals which spent a long time feeding do not necessarily have a high tail beat frequency.

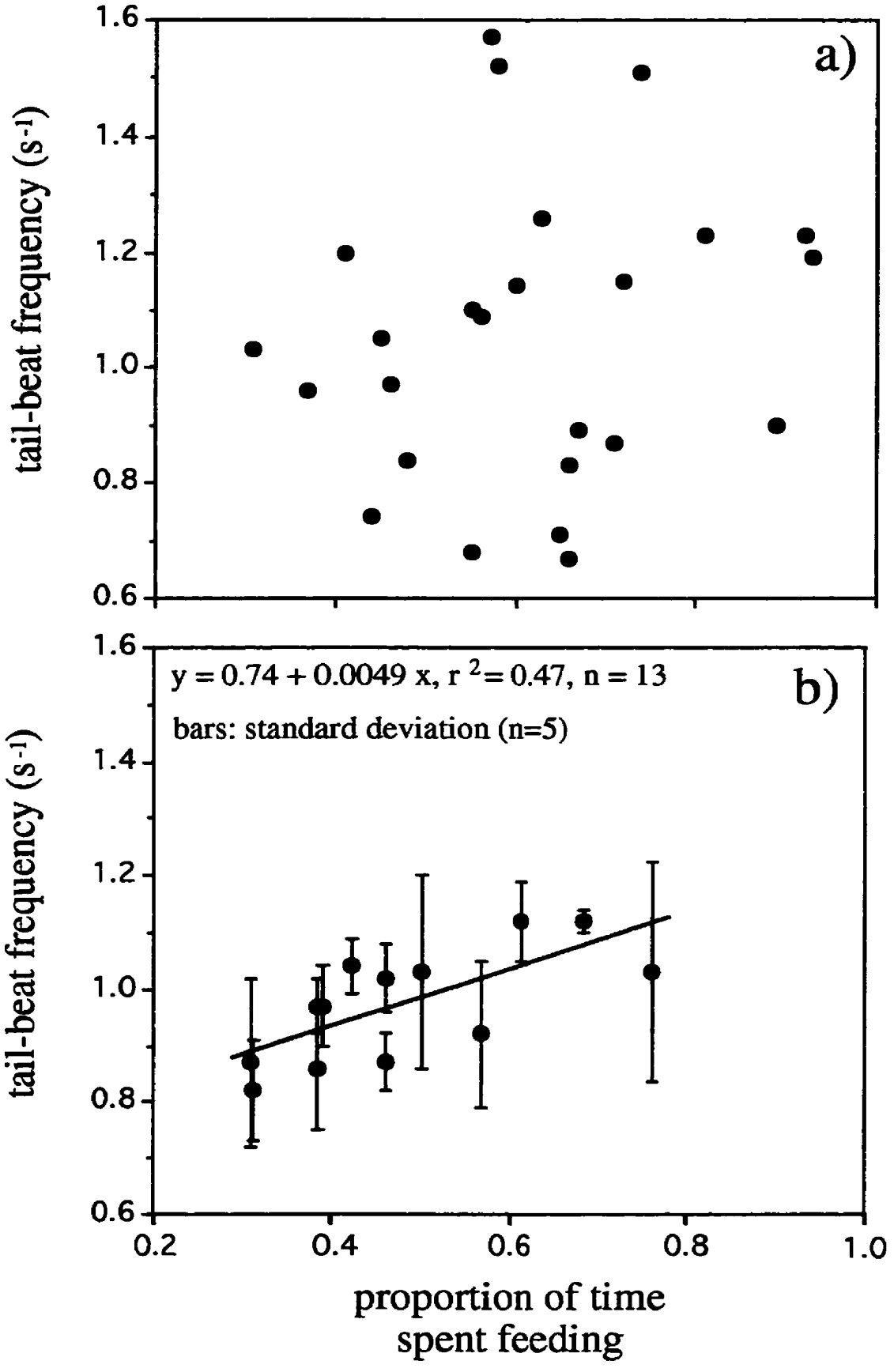

a)
b)
tail-beat frequency (s-1)
1.6
1.4
1.2
1.0
0.8
0.6
y = 0.74 + 0.0049 x, r 2= 0.47, n = 13
bars: standard deviation (n=5)
0.2
0.4
0.6
0.8
1.0
proportion of time
spent feeding

Fig. 1.10: Comparison between proportion of time spent feeding and clearance rate determined in flow-through experiments. About 50% of the variance in clearance rate was explained by time spent feeding. The curved line represents the best least square fit after arcsine transforming the proportion of time spent feeding [$y = -12 + 288$ arc sin $x$; $n = 35$, $r^2 = 0.52$]. The linear regression equation is: $y = -62 + 413\ x$; $n = 35$, $r^2 = 0.51$.

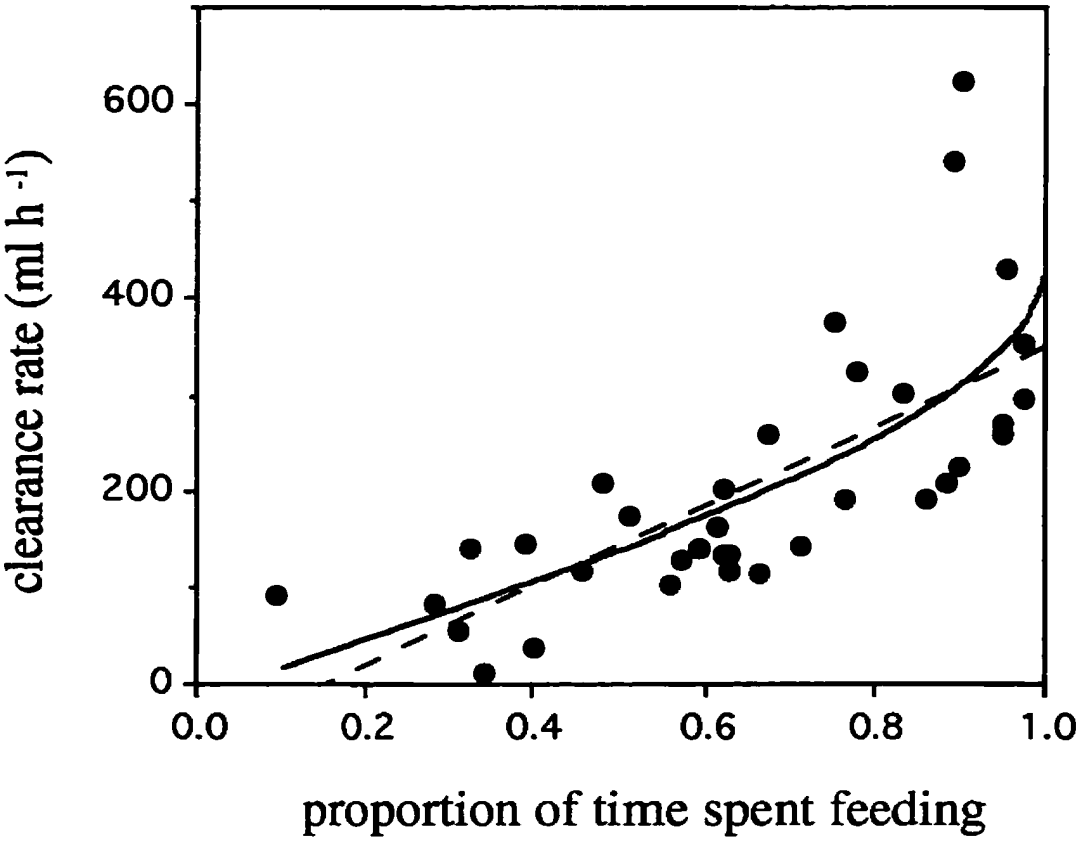

clearance rate (ml h -1)
600
400
200
0
0.0
0.2
0.4
0.6
0.8
1.0
proportion of time spent feeding

Fig. 1.11: Mean time intervals between release of fecal pellets of *Oikopleura vanhoeffeni* fed with diatoms. The average defecation interval and variance increased when time intervals were considered during which the pharyngeal filter was not in place. In these cases, no fecal pellet was formed because no material entered the gut.

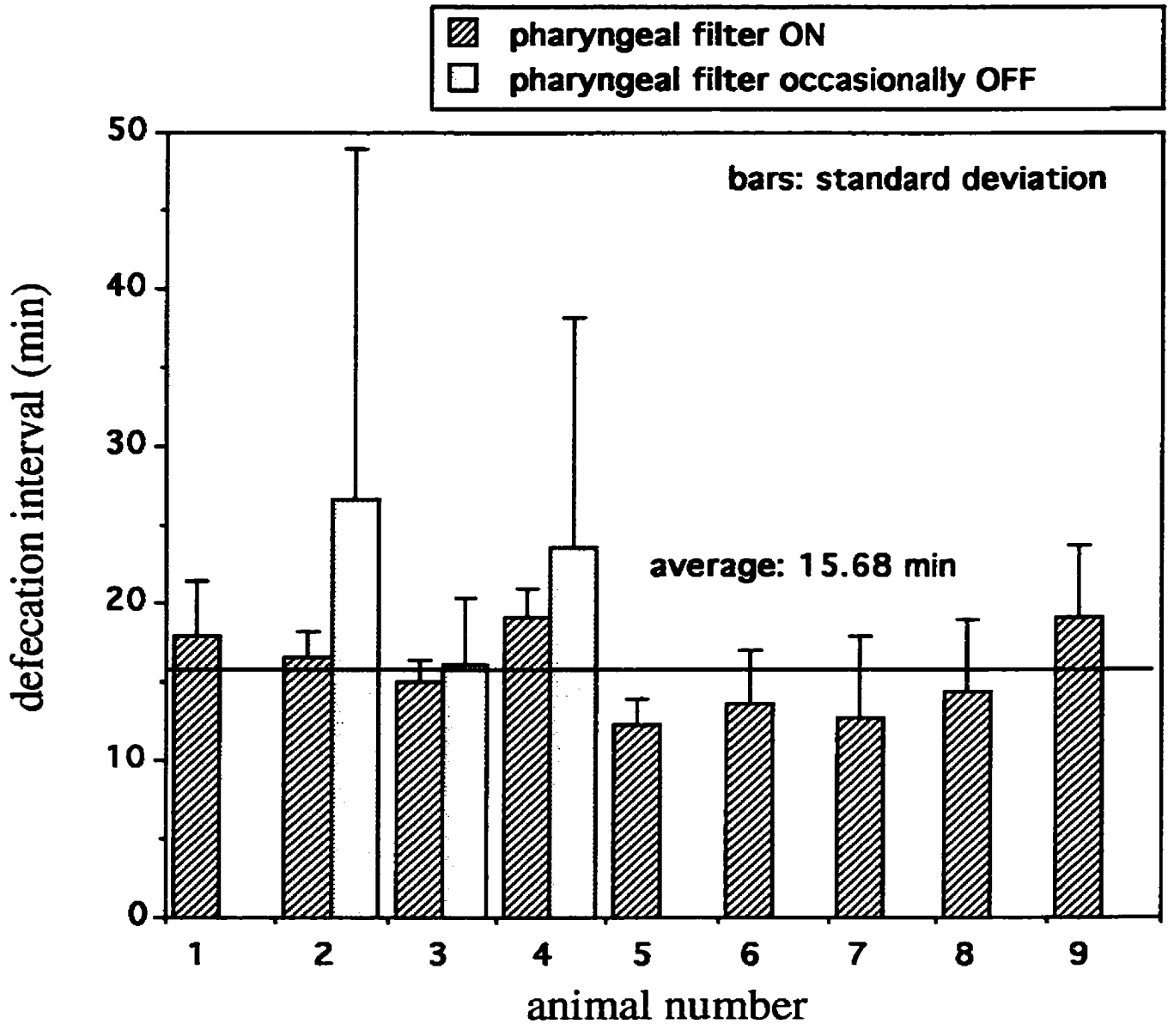

pharyngeal filter ON
pharyngeal filter occasionally OFF
bars: standard deviation
average: 15.68 min
defecation interval (min)
0
10
20
30
40
50
1
2
3
4
5
6
7
8
9
animal number

Fig. 1.12: The defecation intervals of an individual animal did not change even when it was exposed to a range of food concentrations. The outlier at ca. 6 h was due to 'constipation' where one fecal pellet got stuck in the gut and was pushed out by the following pellet. The time interval between these two pellets was therefore less than 3 min.

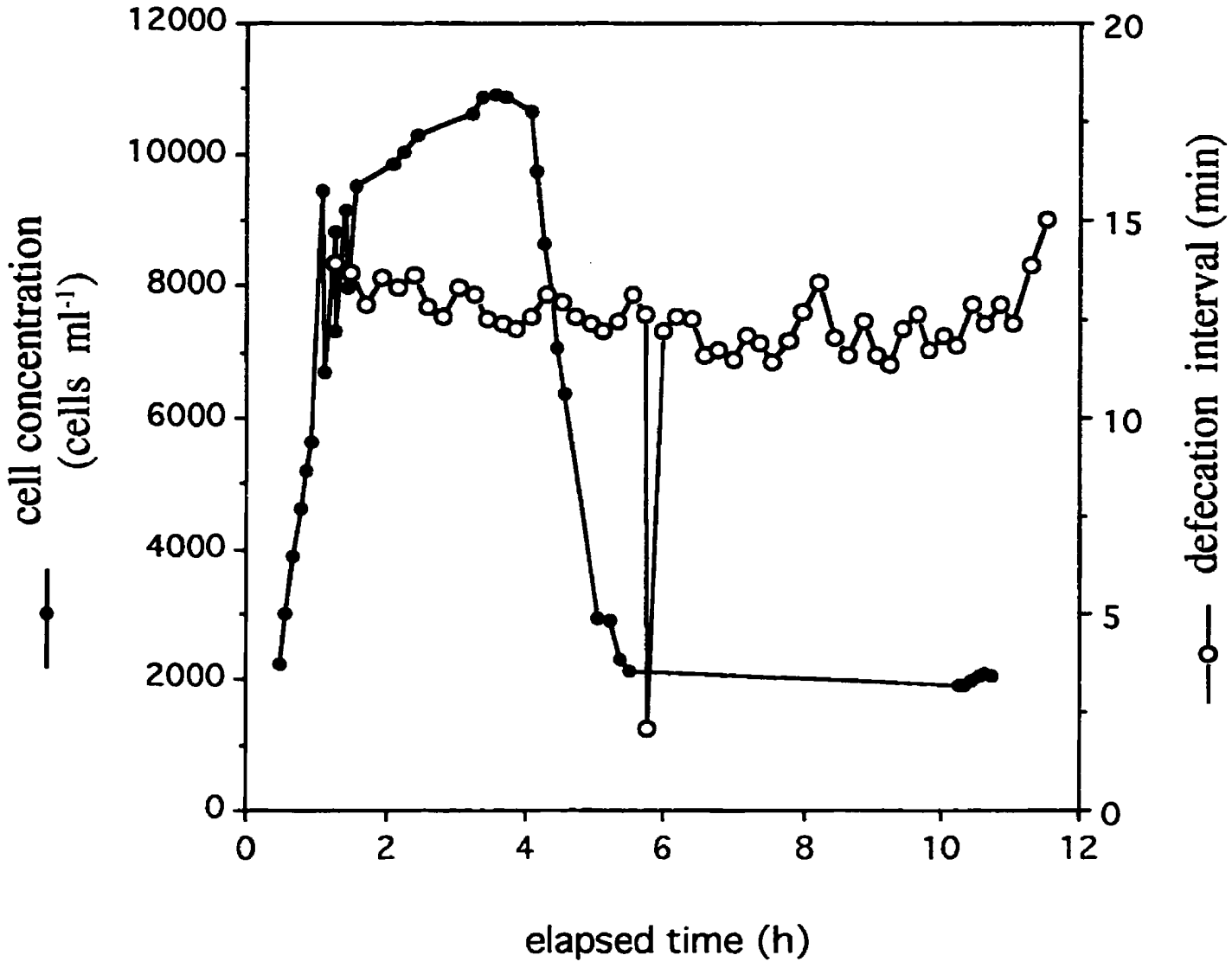
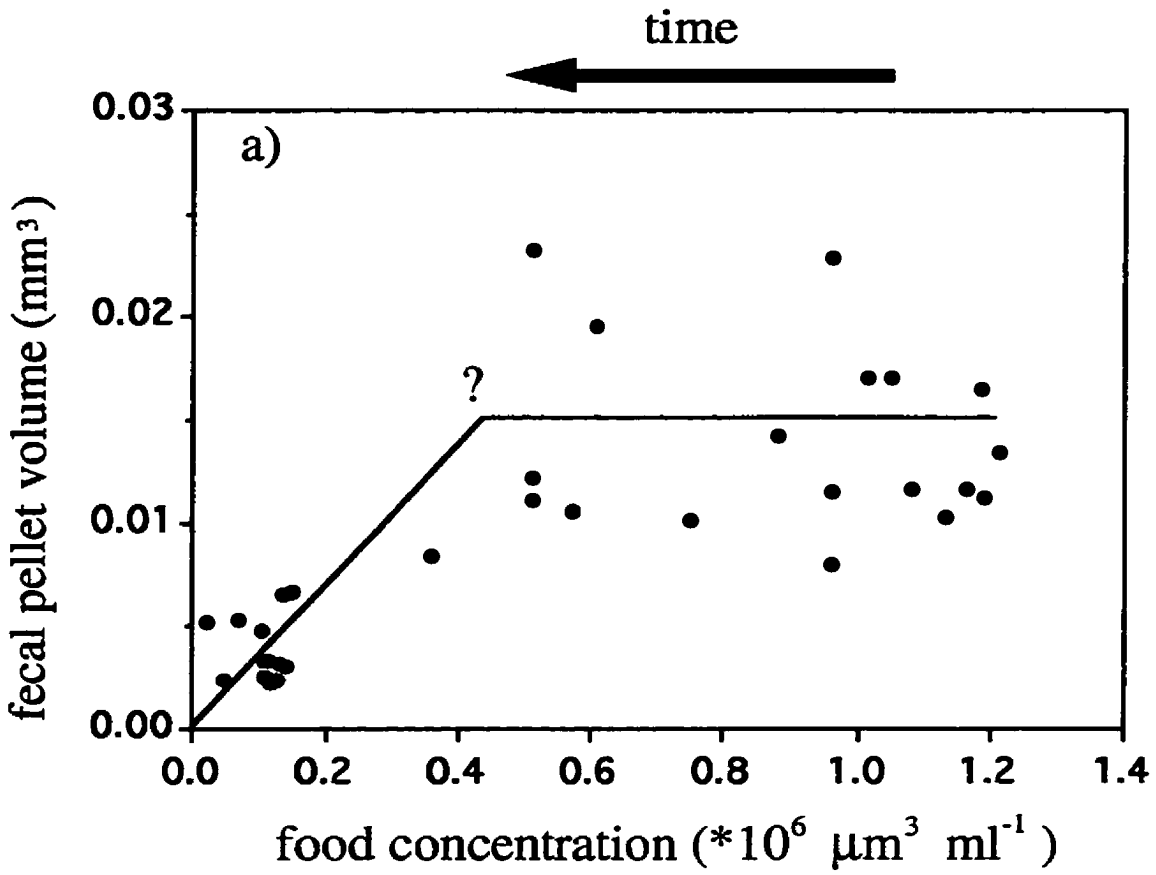
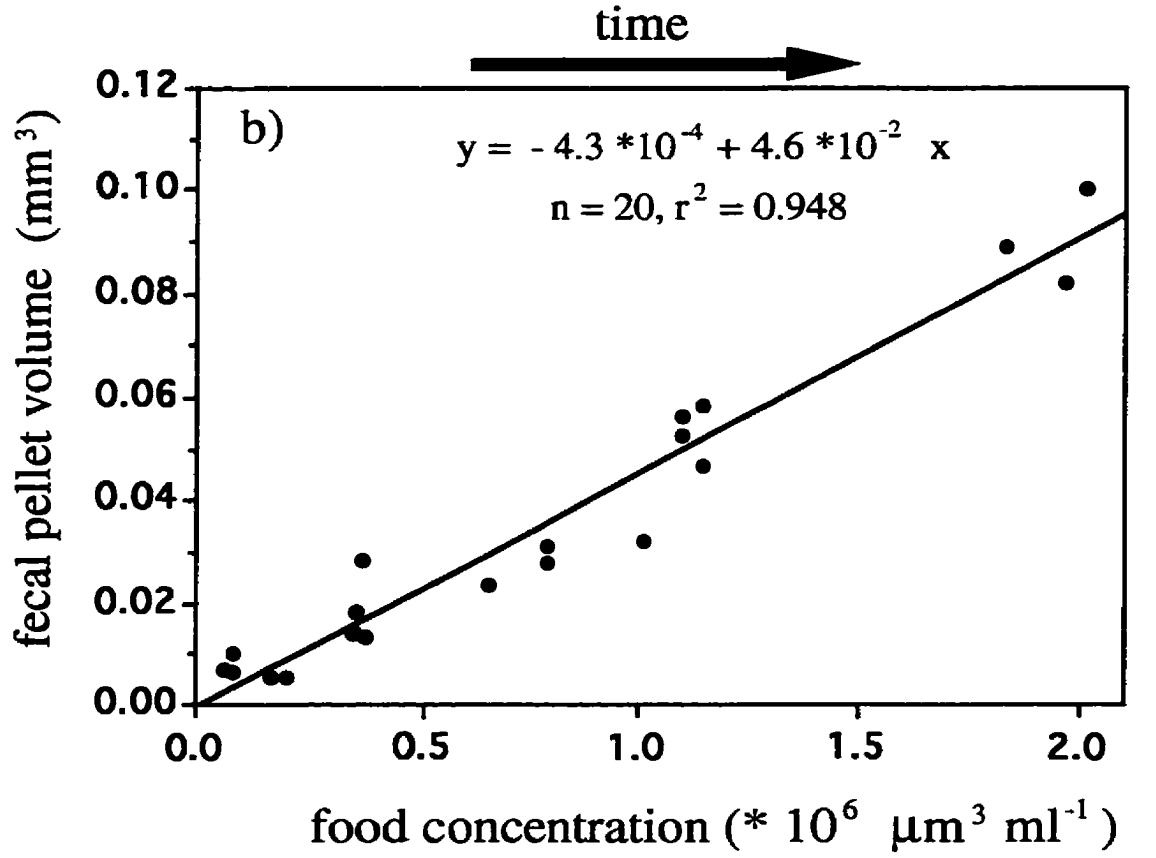

12000
10000
8000
6000
4000
2000
0
20
15
10
5
0
0
2
4
6
8
10
12
cell concentration (cells ml-1)
defecation interval (min)
elapsed time (h)

Fig. 1.13: Examples for two shapes of the functional response in two individuals using fecal pellet volume as a proxy for ingestion rate. (a) shows a saturated ingestion response at ca. 0.4 ppm ($0.4 \times 10^6$ $\mu m^3$ $ml^{-1}$), while (b) shows a condition when saturation did not occur, although the food concentration range was larger in (b) than in (a). The time arrows show that the sequence of low and high concentrations was opposite in the two experiments. The question mark in (a) indicates that the critical concentration could not be determined due to the wide scatter of the data.


time
a)
?
fecal pellet volume (mm³)
0.03
0.02
0.01
0.00
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
food concentration (*10⁶ µm³ ml⁻¹ )


time
b)
y = - 4.3 *10⁻⁴ + 4.6 *10⁻² x
n = 20, r² = 0.948
fecal pellet volume (mm³)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.0
0.5
1.0
1.5
2.0
food concentration (* 10⁶ µm³ ml⁻¹ )

# Chapter 2. Calculation of clearance rate of *Oikopleura vanhoeffeni* from body size and *in situ* behavior. Convergence of various field and laboratory estimates

## 2.1 Introduction

*In situ* observations by SCUBA diving (e.g. Hamner 1975, Alldredge 1976) and submersibles (e.g. Youngbluth et al. 1990) have greatly enhanced our understanding of the biology of gelatinous zooplankton. However, rate processes such as feeding can rarely be estimated directly from observation, and many of the descriptions of zooplankton feeding remain qualitative. Appendicularians may represent an exception to this limitation of *in situ* observations, since their tails, which create water currents through the food concentrating filters, are readily observable. Regular sinusoidal motions of the tail allowed Morris and Deibel (1993) to estimate the flow of water through the tail chamber. Since all the water which passes through the tail chamber must also pass through the food concentrating filter (Flood 1991), the flow rate of water through the tail chamber equals the clearance rate for those particles retained with an efficiency of 100% (Morris and Deibel 1993). It may therefore be possible to calculate clearance rates for oikopleurid appendicularians in the field if their body sizes and pumping behaviors are known.

*Oikopleura vanhoeffeni* is a suitable animal to test the above hypothesis. Firstly, it is relatively large (> 5mm) and therefore observable without any special optical devices,

and the large distance which is possible to maintain between observer and animal in the field allows for observation without disturbance. Secondly, *O. vanhoeffeni* is available in great numbers in the surface waters of the Labrador Current from January to June (during the remainder of the year, the animals are still abundant, but in deeper, colder layers outside the physical limits of divers). Finally, a large amount of feeding rate data for this species has been accumulated in the field and the laboratory which can be compared to behavioral observations. The objectives of this study are therefore to determine whether it is possible to arrive at realistic clearance rates for a population of appendicularians by measuring individual behavioral components, and to examine factors which affect the behavior and therefore the clearance rate of these animals in the field.

## 2.2 Materials and Methods

For the seven *in situ* observation dives, two shore-based divers descended to ca. 8 to 15 m in Logy Bay, the site of the Ocean Sciences Centre (Memorial University, St. John's, Newfoundland, Canada). One diver was equipped with a stop watch, a slate and 16 500-ml wide-mouthed collecting jars. This diver recorded the tail beat frequency of animals upon encounter (not truly random, see legend of Fig. 2.2) by counting the number of beats during a feeding interval determined with a stop watch. A tail-beat was defined as a full cycle from a certain position to the return to the same position (analogous to the crest to crest definition of one wave length). These animals were then collected separately in labeled 500 ml glass jars for later size and stage determination. Three more jars were filled with ambient water to determine the particle concentration over the size range of ingestible particles (i.e. 3 - 64 μm equivalent spherical diameter) with a Coulter Counter

Multisizer II. The lower limit of 3µm used in the Multisizer was close to the pore size of the pharyngeal filter (i.e. 3.3 µm, Deibel and Powell 1987) and the upper threshold close to the mean pore width (70 µm) of the inlet filter (Deibel 1986). The second diver was equipped with two hand counters and recorded the number of feeding and non-feeding animals at first encounter. The proportion of feeding animals in relation to the total number of animals encountered is identical to the proportion of time spent feeding for the average animal (Alldredge 1976). In order to avoid recording from animals disturbed by bubbles or turbulence, the divers swam in large loops and continuously changed the depth of observation. Once the animals were returned to the laboratory, trunk lengths (without gonads, Fig. 2.1) and life-history stages of the animals were determined (Shiga 1976). The life history stages of appendicularians are intervals on a continuum from immature animals without gonads to mature animals carrying ripe eggs and sperms (Shiga 1976).

The clearance rates for particles retained with an efficiency of 100% (which is equivalent to the flow rate through the tail chamber) were calculated using the formula given in Morris and Deibel (1993):

(1) $$cr = w * A * tbf * tsf * 3.6,$$

where 'cr' is the clearance rate (ml $h^{-1}$), 'w' the tail width (mm), 'A' the projected area under the tail ($mm^2$) which is moved along the tail during one tail beat (Fig. 2.1), 'tbf' the tail beat frequency in beats $s^{-1}$, 'tsf' the average time spent feeding (expressed as a dimensionless proportion) and 3.6 a constant which converts seconds into hours and $mm^3$ into ml.

The tail width was calculated from a known trunk length by the equation (Morris and Deibel 1993, unpubl.):

(2) $$w = 0.776\ tl^{1.03}$$

where 'tl' is the trunk length in mm ($n = 23$, $r^2 = 0.91$, Morris and Deibel 1993, unpubl.).

The laterally projected area under the tail which is moved during each tail beat is calculated from trunk length by:

(3) $$A = 3.090\ tl^{1.66},$$

($n = 23$, $r^2 = 0.85$, Morris and Deibel 1993, unpubl.).

The clearance rates calculated according to equation 1 and using the data from the field observations were compared with published data referenced in Table 2.1. Briefly, in the 'static experiments' (chapter 1), clearance rates were determined by repeated sampling of the exponentially decreasing particle concentration in 500 ml jars. The food cells, either *Thalassiosira pseudonana* or *T. nordenskioldii*, were counted with a Coulter Counter Multisizer II. The grazing coefficient (g) is the slope of a linear regression fitted through natural-log transformed particle concentrations over time. Multiplied by the jar volume, g can be translated into clearance rates (Frost 1972).

For the flow-through experiments, a novel flow-through device was designed (chapter 1). Clearance rates were calculated using the formula:

(4) $$cr = fr * (C_i - C_o)/C_o,$$

where fr is the flow rate in ml $h^{-1}$, $C_i$ and $C_o$ the inflow and outflow concentrations in cells $ml^{-1}$, respectively.

In Deibel (1988) and Knoechel and Steel-Flynn (1989), clearance rates were calculated using particle uptake by the house and the animals over periods of time shorter than the gut passage times of the animals (< 5min in Deibel 1988 and 10 min in Knoechel and Steel-Flynn 1989). The tracer particles consisted either of fluorescent latex microspheres (Deibel 1988) or $^{14}C$ labeled *Scenedesmus gracilis* (Knoechel and Steel-Flynn 1989). The following formula was used to calculate clearance rates:

(5) $$cr = (n_H + n_A)\, c^{-1}\, t^{-1},$$

where $n_H$ and $n_A$ are the number of latex beads or dpm of $^{14}C$ in the house and animal, respectively, 'c' is the concentration of latex beads in the suspension or dpm per volume water and 't' the incubation time.

Clearance rates were also calculated by measuring chlorophyll *a* in the guts of field-collected animals (chapter 3):

(6) $$cr = n_A\, f\, gpt^{-1}\, c^{-1},$$

where $n_A$ is the amount of chl *a* in the animal (ng $ind^{-1}$), f the correction factor for chl *a* conversion, gpt the gut passage time (h), and c the concentration of chl *a* in the water (ng $ml^{-1}$). The values for gut passage time and the correction factor for chl *a* conversion were taken from chapter 3. Chl *a* conversion was defined as the conversion of chl *a* into its fluorescent and non-fluorescent breakdown products and was on average 79% (i.e. the values had to be corrected by multiplying by 4.76). The average gut passage time was 0.8 h (chapter 3).

Original data from Deibel (1988) were used, whereas the values from Fig. 4 of Knoechel and Steel-Flynn (1989) were digitized. Replotting of these data gave identical parameters as estimated by the authors. For all comparisons, a size range from 3 - 4 mm trunk length only was considered, which was a size range well covered in all experiments and observations.

**2.3 Results**

Fig. 2.2 presents the mean lengths at each maturity stage of animals encountered on six of the seven dives. On one dive (i.e. 9 May 1995), trunk length and stages were not determined. Stage 4 was the most commonly encountered. Table 2.2 summarizes temperature, food concentration, average tail beat frequency and proportion of time spent feeding for the *in situ* observations. The temperature during the seven dives ranged from -1.1 to + 3.8 °C. The average tsf for all dives was 0.60 and lay between 0.47 to 0.77 (< 2 fold). Similarly, the tail beat frequency varied little among dives (means 1.10 to 1.56 $s^{-1}$). The proportion of time spent feeding was not significantly affected by trunk length (n=6,

p=0.15), temperature (n=7, p=0.81) or particle concentration (n=6, p=0.39). Since the number of measurements for time spent feeding was low, randomizations were performed to confirm the obtained p values from the parametric statistics (Manly 1991). The p-values calculated using randomization were identical to, or only slightly different from, those obtained using the standard F-distribution. Therefore, the assumptions for the parametric analysis were met and the p-values from the parametric tests were reported. Since time spent feeding was not affected by trunk length, an overall mean of 0.60 was calculated for the seven dives for use in the model. Tail beat frequency was inversely correlated with trunk length, a relationship which is described by the regression equation given in Fig. 2.3a. The residuals of this regression were significantly correlated with temperature and particle concentration (Fig. 2.3b and c). Figure 2.4 shows how the clearance rates calculated from field observations, in combination with the Morris and Deibel (1993) model (equations 1, 2 and 3), compare with the data collected in laboratory and field experiments (Table 2.1). Since tail beat frequency is only one of four components in the estimation of clearance rate, the variance caused by differences in tail beat frequencies is a minimum estimate of the expected total variance among individuals.

The clearance rates determined by static, flow-through experiments and the model approach were not significantly different (Fig. 2.4) using the GT2 technique ($\alpha = 0.05$) for unequal sample sizes (Sokal and Rohlf 1981). There were also no significant differences among the means of clearance rates from static and flow-through experiments on one hand and the mean clearance rate determined by Deibel (1988) on the other. However, the clearance rates calculated from the gut pigment technique and those determined by Knoechel and Steel-Flynn (1989) and Deibel 1988 were significantly lower than those predicted by the model (Fig. 2.4).

## 2.4 Discussion

### Influence of environmental variables on feeding behavior

The field observation data covered most of the natural range of temperatures in which *O. vanhoeffeni* can be found. *O. vanhoeffeni* is stenothermic and occurs at temperatures as low as minus 1.5 but rarely at temperatures higher than 5 °C. Considering the weak temperature effect (Fig. 2.3b), tail beat frequency and therefore feeding rates would change only slightly over the entire range of temperatures. Approximately 45% of the variance in tail beat frequency can be explained by the multiple regression model:

(7) $$tbf = 1.70 - 0.19\ tl + 0.07\ temp + 0.57\ conc,$$
$$[R^2 = 0.45,\ n = 62,\ p < 0.0001]$$

where 'temp' is the temperature (°C) and 'conc' the particle concentration between 3 and 70 μm (ppm). However, since trunk lengths were not measured on 9 May 1995, the residual plot is incomplete. Considering that the particle concentration on 9 May was the highest of all dives and tail beat frequency was low (Table 2.2), the significant positive relationship between tail beat frequency and particle concentration as shown in equation 7 has to be viewed with caution. Furthermore, an inverse relationship between tail beat frequency and particle concentration was apparent in controlled laboratory experiments (chapter 1). In those experiments, a 100-fold increase in food concentration resulted in a decrease in the tail beat frequency of about 2-fold. Since the entire range of food concentrations in the field was only ca.10-fold over all dives (i.e 0.11-1.5 ppm), I would

not have expected an observable effect. By excluding particle concentration from the multiple regression model one would therefore arrive at a more realistic equation:

(8) $$tbf = 1.9 - 0.19\ tl + 0.07\ temp$$
$$[R^2 = 0.34, n = 76, p < 0.0001]$$

The proportion of time spent feeding was not influenced by trunk length, temperature and food concentration. The overall mean of time spent feeding of 60% is very close to the mean of 51% reported for the same species in Deibel (1988). Smaller oikopleurid appendicularians examined in the Gulf of California and in the Florida current display a wide range of time spent feeding (Alldredge 1976). *Megalocerus huxleyi*, *Stegosoma magnum*, *O. rufescence* and *O. longicauda* filtered between 89% and 100% of the time, whereas *O. intermedia* and *O. cornutogastra* filtered 59% and 25 % of the time, respectively (Alldredge 1976). *O. fusiformis* did not feed at first encounter (Alldredge 1976). The observed proportions of time spent feeding by *O. vanhoeffeni* are therefore in the middle of the range and close to those for *O. cornutogastra*.

Why is the allometric exponent smaller than 3?

From Fig. 2.3a it is apparent that smaller individuals of *O. vanhoeffeni* have a higher average tail beat frequency than larger ones. This size dependent change of behavior means that the volume of water filtered does not increase with the cube of the trunk length. Calculating clearance rate from the size-dependent tail beat frequency (Fig. 2.3a) and regressing it against trunk length results in an allometric exponent of b = 2.2. An exponent lower than 3 has already been determined empirically by Deibel (1988, b

=1.8) and Knoechel and Steel-Flynn (1989, b = 1.6) for appendicularians, and also for salps ranging from b = 1.4 to 2.1 (Madin and Cetta 1984). There are several possible explanations for this relation. First, the underlying observation agrees with the general observation that metabolic rate scales with weight to the power of 0.75, not 1, even in invertebrates (Schmidt-Nielsen 1984). On average, a smaller animal would therefore have to pump faster in order to collect the required amount of food. An exponent lower than 3 also agrees with the observation that weight-related variables such as carbon or nitrogen content frequently scale with body length with an exponent smaller than 3 in pelagic tunicates. For instance, an average exponent of 2.3 has been reported by Huntley et al. (1989) for salps. This relationship also holds true for some appendicularians, such as *O. labradoriensis* (2.86 for carbon and 2.83 for nitrogen, Riehl 1993) and *O. dioica* (2.63 for carbon, King et al. 1980). In *O. vanhoeffeni*, however, body carbon is related to body length by a power function of 3.1 and 3.2 as reported by Deibel (1986) and Riehl (1993), respectively. In the same studies, nitrogen scaled vs. body length with an exponent between 2.64 and 2.85 (Deibel 1986, Riehl 1993).

## Comparison of clearance rates

The most detailed laboratory experiments in which the clearance rates of *O. vanhoeffeni* were examined are the static and flow-through experiments (Table 2.1, Fig. 2.4). Due to repeated measurements on individuals, analytical error could be kept to a minimum so that most of the remaining variance is attributable to the variability within and among individual animals (chapter 1). It is therefore very encouraging that the estimated clearance rates calculated from the Morris and Deibel (1993) model for animals observed in the field agree so well with these laboratory studies.

The clearance rates derived from the gut pigment technique are significantly lower than those derived from static experiments, flow-through experiments and *in situ* behavior (Table 2.2). This result can at least partly be explained by the fact that the gut pigment technique considers particles which are ingested by the animals only and not those trapped in the house. All the other experiments consider total particle removal (house + animal). Another reason for the low values from the gut pigment technique is that an overall correction of 4.76 was used, which is a conservative estimate of pigment destruction (chapter 3). However, for Fig. 2.4 I decided to show the lowest values, to cover the maximum range. Finally, animals were collected randomly, thus including non-feeding animals with very little gut content. In contrast, the laboratory measurements were all performed on feeding animals. When the animal switches off the pharyngeal filter it is not ingesting algae, although the total clearance rate does not change. Clearance rates based on measurements of gut fullness are therefore always lower than total clearance rates.

The clearance rates determined by Knoechel and Steel-Flynn (1989) were significantly lower than those measured in the static experiments and the model. However, due to the high variability and the small sample size for animals between 3 and 4 mm trunk length in Knoechel and Steel-Flynn (1989), there were no significant differences between the clearance rates from Knoechel and Steel-Flynn (1989) and those from the flow-through experiments in the present study, the clearance rates reported by Deibel (1988) and the gut pigment data from the present study. The values by Deibel (1988) seem to lie in the middle of the range, being significantly different only from the model estimates. Again, it is important to note that the variance of the model estimates is

only a minimum estimate of the total variance, since tail beat frequency is only one factor in the model. If the total behavioral variance were known, the model data may not be statistically distinguishable from the other estimates.

The values obtained from the gut pigment technique and those reported by Deibel (1988) and Knoechel and Steel-Flynn (1989) were obtained using natural sea water. Like the gut pigment values, they represent a 'snapshot' of appendicularians in houses of different ages, because of the short incubation time. In contrast, the static and flow-through experiments were performed using animals in relatively new houses and lab cultures of diatoms in filtered sea water (chapter 1). Since clogging of the food concentrating filter has a significant effect on the measured clearance rates (chapter 1), the difference between the field and the laboratory data may be attributable to this very factor.

Problems of the model approach

One of the major problems with the model approach is the peculiar functional feeding response of appendicularians (chapter 1). Clearance rates are reduced when food concentration increases, but also decrease with the age of the house. This means that the house deteriorates and that the condition of the house has a significant effect on the observed clearance rates. At times of high seston concentrations, such as during the spring diatom bloom, the estimated rates from the behavior model will be significantly higher than the true clearance rates (chapter 1). Another problem during the spring diatom bloom is that large, chain-forming diatoms are abundant which cannot pass through the inlet filter mesh. Not only does this make the upper threshold of particle size ambiguous when ingestion rates are calculated, but these diatoms may also clog the inlet filters and thereby

further reduce clearance rates. However, since for most of the year ingestible carbon concentrations are below 100µg C $l^{-1}$ and few chain-forming diatoms are present (Deibel 1988), this problem is probably not severe on an annual basis (chapter 1).

Although the number of non-feeding animals was not estimated, their number was considerable. In general, an animal is not filtering water when it is either outside its house or when its trunk is detached from its house (i.e. not able to push water through the house; chapter 1). Using size-based clearance rate estimates alone, one would not account for the unknown proportion of non-feeding animals and would therefore overestimate the true population clearance rate.

The problem of particles trapped in the house for estimating carbon flux

Not all filtered particles reach the mouth of the animals. Some of them become attached to the filter surface and consequently reduce the efficiency of the filter (chapter 1). The only estimate to date for the proportion of particles permanently trapped in the filter was published by Gorsky et al. (1984), who determined that ca. 30% of the filtered particles remain in the house after five hours of feeding by *O. dioica*. This issue is very important, considering the contribution of appendicularians to the overall vertical flux of particles through the water column (Taguchi 1982). Particles which have not been ingested have a much higher nutritive value than the more refractory fecal pellets, and may be of importance for the thriving communities in abandoned houses (Steinberg et al. 1994).

Notwithstanding the importance of this issue, it is not straightforward to obtain an estimate of how much POC remains trapped in the house. Predictably, the degree to which certain particles stick to the filter depends on their types and sizes. Since the gut pigment technique in Fig. 2.4 is the only estimate of clearance rates which is based on the ingestion of particles by the animals alone, Fig. 2.4 can be used to arrive at minimum to maximum estimates of particles stuck to the house. When the clearance rate values from the gut pigment technique are compared to the data set of Knoechel and Steel-Flynn (1989), the loss of particles in the house may be as little as 15%, but when compared to the model it may be as much as 300%, which illustrates that further investigations are needed to understand how much of the filtered POC remains undigested in discarded appendicularian houses.

Significance of the model approach

Despite the variety of techniques and investigations which produced the results in Fig. 2.4, the convergence of rates within a 3-fold range is very promising, especially since a large portion of this discrepancy can be explained. The model approach paints an ideal picture with maximum physiological rates which are compromised by the reality of clogging filter surfaces, the peculiarity of the functional feeding response and simply that a certain proportion of animals does not feed at any given time. The convergence of theory, observation and experiment gives confidence in the various experimental approaches and our concept of the filtering behavior of appendicularians. Although detailed *in situ* feeding studies in response to various environmental conditions cannot be

replaced by the model approach, an approximate estimate of total clearance rates is useful for appendicularians for which it is difficult to obtain empirical feeding data, such as the giant, deep sea appendicularian *Bathochordaeus* sp., or for historical data for which only abundance and size distributions of appendicularians exist.

Table 2.1. Summary of the data collections used in this study. Chapter 1 and chapter 3 used two different techniques. One was concerned with the application of the gut pigment technique for *O. vanhoeffeni* in the field (chapter 3), the other one (chapter 1) tested how food concentration would influence the feeding behavior and clearance rates in controlled laboratory studies. See chapter 1 for details on the experimental set up of the static and flow-through experiments. '†' values calculated using a factor of 60 to convert chl *a* (µg $l^{-1}$) into µg C $l^{-1}$. *T. nord.* = *Thalassiosira nordenskioldii*, *T.pseud.* = *Thalassiosira pseudonana*, *Scened.* = *Scenedesmus quadricauda*.

| authors | type of experiment | marker | compartments | food | food concentration range (µg C $l^{-1}$) | incubation time |
|---|---|---|---|---|---|---|
| chapter 1 | static | diatom cells | house & animal | *T. nord.* *T. pseud.* | 1.5 - 1500 | 5 hours |
| | flow-through | diatom cells | house & animal | *T. nord.* *T. pseud.* | 1.8 -100 | > 2 h |
| chapter 3 | gut pigment | natural chlorophyll | animal | natural seawater | 3 - 118 † | - |
| Knoechel and Steel-Flynn 1989 | marker uptake | $^{14}C$ labelled *Scened.* | house & animal | natural seawater & *Scened.* | 10 - 20 | 10 min |
| Deibel 1988 | marker uptake | latex microspheres | house & animal | natural seawater | 29 - 85 | ca. 3 - 5 min |

Table 2.2. Behavior of *Oikopleura vanhoeffeni*, and temperature and particle concentration, during 7 *in situ* observations in the Labrador Current, Newfoundland. 'Particle concentration' refers to the volume of particles in a size range from 3 to 64 μm as determined by a Coulter Counter Multisizer II.

| date | temperature (°C) | particle concentration (ppm) | mean trunk length (mm) | tail beat frequency ($sec^{-1}$); ±SD; n | proportion of time spent feeding; ±SD; n |
|---|---|---|---|---|---|
| 30 May 1994 | + 3.3 | n.d. | 3.69 | 1.39 (± 0.26; 15) | 0.60 (± 0.21; 9) |
| 8 June 1994 | + 1.1 | 0.11 | 4.05 | 1.10 (± 0.24; 12) | 0.77 (± 0.04; 4) |
| 9 May 1995 | 0.0 | 1.09 | n.d. | 1.13 (± 0.23; 15) | 0.47 (± 0.11; 7) |
| 24 May 1995 | + 1.0 | 0.62 | 3.25 | 1.52 (± 0.19; 14) | 0.73 (± 0.13; 11) |
| 2 June 1995 | + 1.8 | 0.36 | 2.54 | 1.45 (± 0.44; 14) | 0.53 (± 0.20; 11) |
| 14 June 1995 | + 3.8 | 0.26 | 2.90 | 1.56 (± 0.38; 14) | 0.58 (± 0.20; 10) |
| 12 March 1996 | - 1.1 | 0.18 | 3.40 | 1.10 (± 0.27; 13) | 0.55 (± 0.14; 20) |
| column average | + 1.4 | 0.45 | 3.30 | 1.32 | 0.60 |

## 2.6 Figures

Fig. 2.1. Lateral and dorsal views of *Oikopleura vanhoeffeni*. 'A' is the lateral projectional area of the volume of water which is moved by one tail beat. 'w' is the width of the tail. Both 'A' and 'w' can be calculated from a known trunk length using equations 2 and 3 (see text). The trunk length was measured from the mouth opening to the posterior margin of the gut (excluding the gonads).

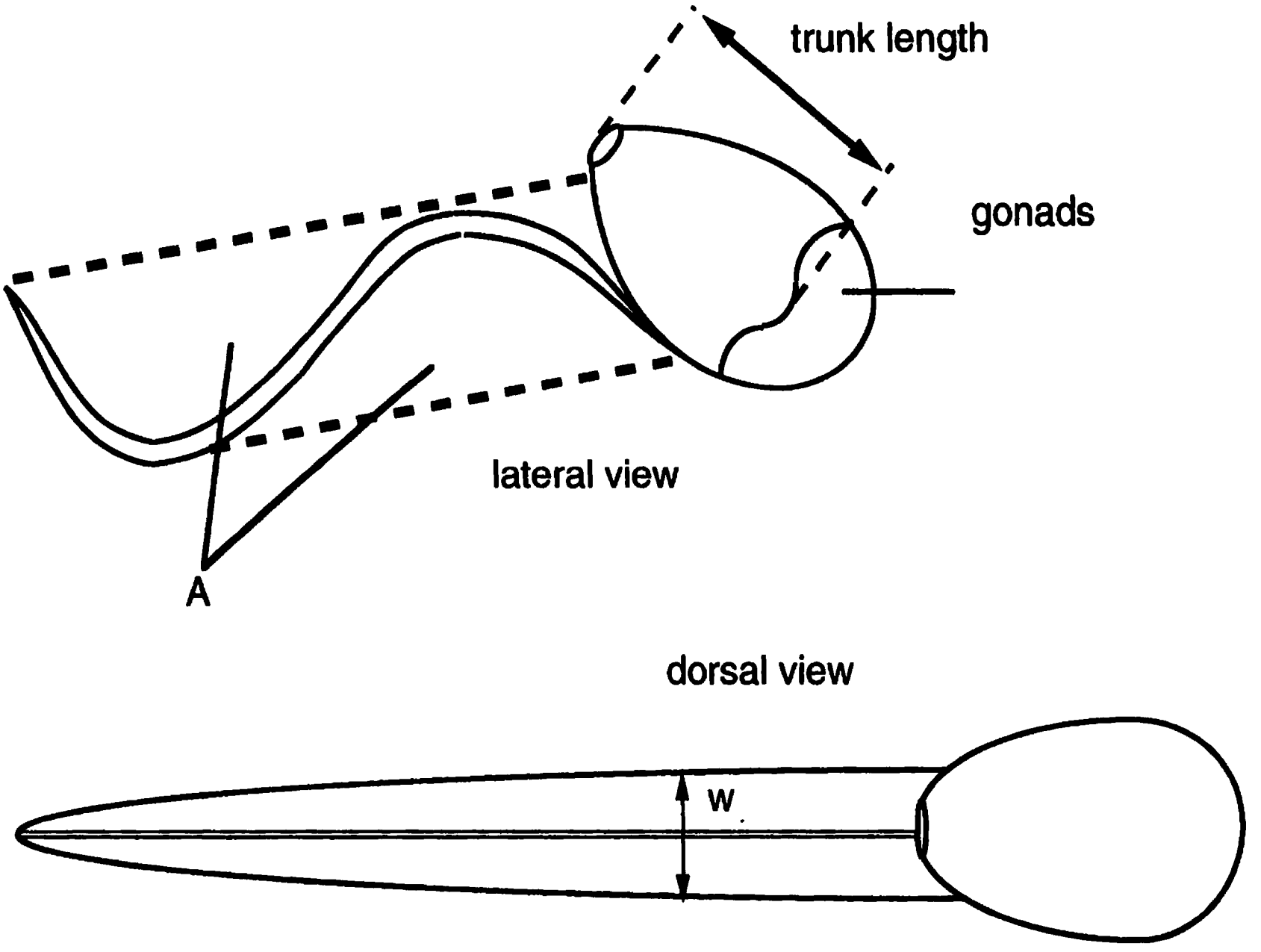

trunk length
gonads
lateral view
A
dorsal view
w

Fig. 2.2. Comparison of trunk length and maturity stages (Shiga 1976) of animals observed in the field. The average size of animals increases with the maturity stage, but there is a wide variability of trunk lengths within each stage. Note that the frequency of animals at each stage is not representative of the actual frequency distribution of stages in the field. It is biased by the perception skill of the diver since larger animals have a higher probability of getting collected than smaller ones. Bars: standard deviation.

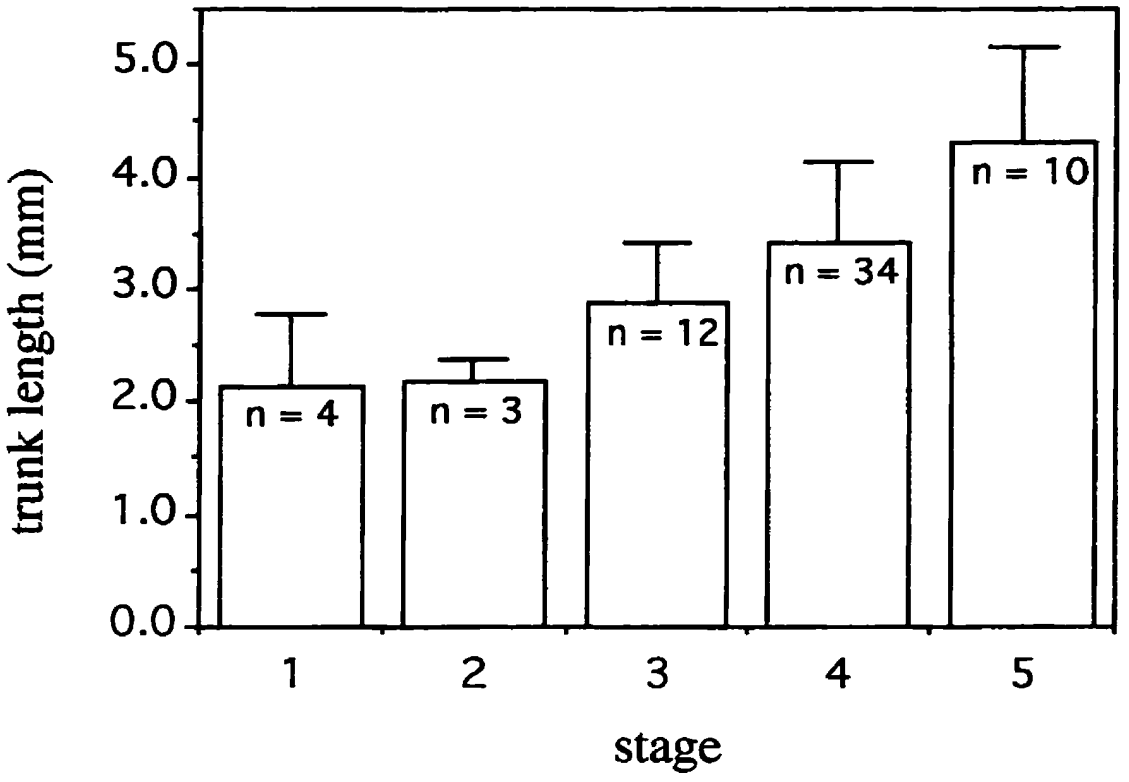

trunk length (mm)
5.0
4.0
3.0
2.0
1.0
0.0
n = 4
n = 3
n = 12
n = 34
n = 10
1
2
3
4
5
stage

Fig. 2.3. Tail beat frequencies in response to trunk length (a), and the residuals (res 1) of this regression against temperature (b). The residuals of the multiple regression using trunk length and temperature from equation 8 (res 2) were regressed against particle concentration in (c). Abbreviations: tbf = tail beat frequency ($sec^{-1}$), tl = trunk length (mm), temp = temperature (°C), conc = particle concentration (ppm).

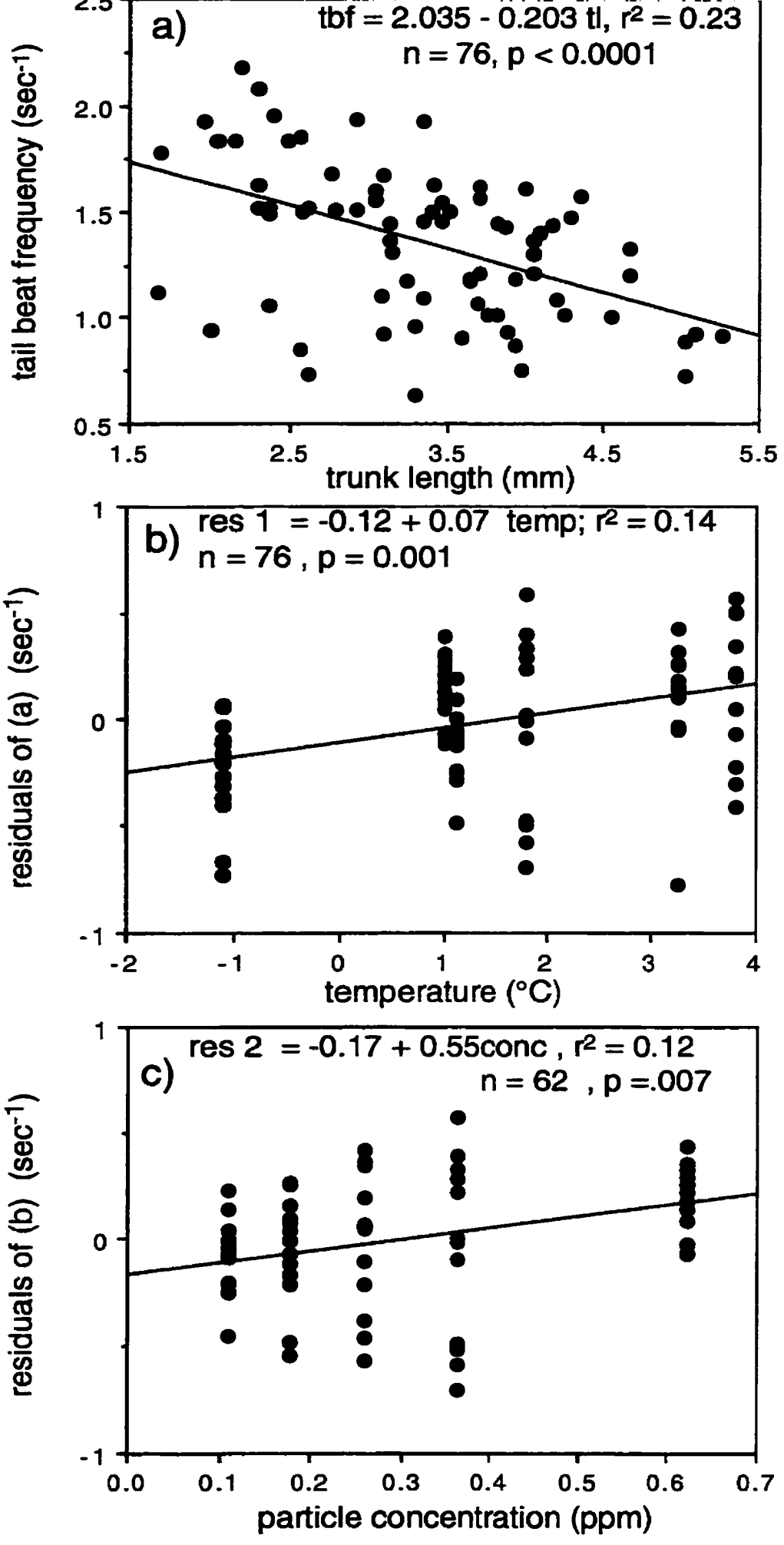
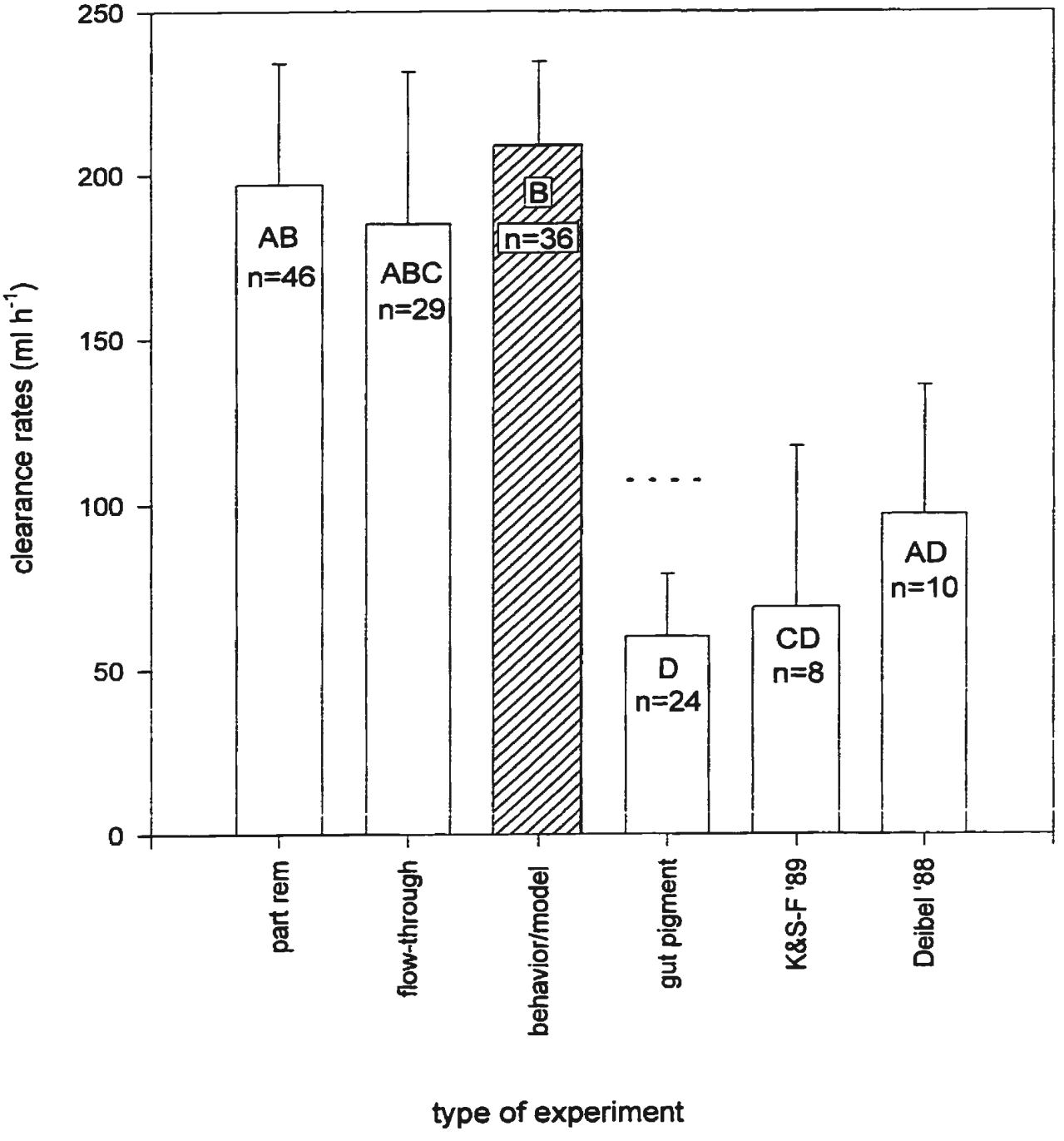

a)
tbf = 2.035 - 0.203 tl, r² = 0.23
n = 76, p < 0.0001
tail beat frequency (sec⁻¹)
2.5
2.0
1.5
1.0
0.5
1.5
2.5
3.5
4.5
5.5
trunk length (mm)
b)
res 1 = -0.12 + 0.07 temp; r² = 0.14
n = 76 , p = 0.001
residuals of (a) (sec⁻¹)
1
0
-1
-2
-1
0
1
2
3
4
temperature (°C)
c)
res 2 = -0.17 + 0.55conc , r² = 0.12
n = 62 , p =.007
residuals of (b) (sec⁻¹)
1
0
-1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
particle concentration (ppm)

Fig. 2.4. Comparison of clearance rates measured by five different approaches listed in Table 2.1. static = static experiments; flow-through = flow through experiments; behavior/model = clearance rates calculated using size and tail beat frequency of field collected animals; gut pigment = clearance rates as determined by a collection of animals and water in Logy Bay in spring 1995; K&S-F '89 = data digitized from Fig. 4 in Knoechel and Steel-Flynn 1989; Deibel '88 = data taken from Fig. 1 in Deibel (1988). Animals in the size range from 3 to 4 mm only were considered since this size range was covered by all experiments. Bars are 95% confidence limits. The dashed line indicates the mean clearance rate for the gut pigment technique if a variable correction factor is used as suggested in chapter 3 and if one assumes that 30% of filtered particles are trapped in the house (Gorsky et al. 1984). If columns share the same letter they are not significantly different from each other at the $\alpha = 0.05$ level according to the GT2 multiple comparisons among means test. This test is preferred for unplanned comparisons and unequal sample sizes (Sokal and Rohlf 1981).


250
200
150
100
50
0
clearance rates (ml h-1)
AB
n=46
ABC
n=29
B
n=36
D
n=24
CD
n=8
AD
n=10
part rem
flow-through
behavior/model
gut pigment
K&S-F '89
Deibel '88
type of experiment

# Chapter 3. Pigment destruction and gut passage time. Testing the assumptions of the gut pigment technique for *Oikopleura vanhoeffeni*

## 3.1 Introduction

Feeding activity can be quantified by examining the amount of food in the gut, and stems from the general observation that gut fullness reflects feeding activity (Bajkov 1935). A marker pigment, such as chl *a*, is an obvious choice as a measure of gut fullness for herbivorous invertebrates, since it can be measured with high precision, requires a minimum of analytical effort and is a proxy for phytoplankton biomass. The gut pigment technique, as introduced by Mackas and Bohrer (1976) for marine copepods, has found wide application in zooplankton ecology because it has allowed for high numbers of replicates and therefore for high spatial and temporal resolution in estimating herbivorous grazing activity. The analytical simplicity of this method makes it suitable for shipboard use, yielding estimates of feeding activity in the field which are free of the problems associated with laboratory experiments. However, three fundamental conditions must be met in order for the gut pigment technique to give information beyond the amount of undegraded pigment in the gut: 1) the tracer must be conservative or it must be possible to reconstruct its original (i.e. ingested) amount, 2) the gut passage time must be constant or

known for all levels of ingestion and 3) the tracer must be equally distributed among the food items or, alternatively, the process of ingestion must be non-selective. The applicability of the gut pigment technique in any given situation will therefore depend largely on the mechanisms of feeding and digestion, and one can expect that not all zooplankton are equally suited for this technique. For copepods, in which the gut pigment technique has found wide application, all three assumptions are generally violated. Reconstruction of the original amount of chl *a* is difficult, if not impossible, since the degree of destruction is dependent on unknown factors such as recent feeding history (Penry and Frost 1991). The variability of gut passage times, which may change exponentially with the amount of food in the gut (Dagg and Walser 1987), and the high degree of selectivity and omnivory (e.g. Cowles et al. 1988, Vanderploeg 1990, Kleppel et al. 1991) impose additional constraints on the gut pigment technique when applied to copepods. Consequently, low levels of chlorophyll in the gut of these animals could either mean low ingestion rates or high preference for heterotrophic food. The non-selective nature of food uptake by pelagic tunicates eliminates the latter and most serious of these problems, rendering pelagic tunicates good candidates for successful application of this technique. More importantly, the gut pigment technique seems to be one of the few choices remaining to estimate levels of feeding in some pelagic tunicates which cannot be maintained under experimental conditions (Madin and Kremer 1995).

Globally, pelagic tunicates constitute a large fraction of the biomass and production of the zooplankton community (Udvardy 1954, Fortier et al. 1994, Hopcroft

and Roff 1995). They are capable of removing particles as small as colloids from the water column and pack them into large, rapidly sinking fecal pellets, thereby contributing significantly to the flux of carbon (Michaels and Silver 1988, Flood et al. 1992, Urban et al. 1992, Bedo et al. 1993). In some systems, pelagic tunicates equal (Knoechel and Steel-Flynn 1989) or exceed the grazing impact of the entire copepod population (Nakamura et al., in press). During bloom conditions, pelagic tunicates compete with other major zooplankton for food (e.g. euphausiids, Nishikawa et al. 1995) and significantly change entire phytoplankton distributions (Deibel 1985, Zeldis et al. 1995, Deibel et al., in prep.). In a seasonal study of the abundance and gut pigment content of the zooplankton community in the Southern California Bight, Landry et al. (1994) found that appendicularians (*Oikopleura* sp.) exceeded copepods in terms of their community gut pigment values (i.e. population abundance * gut content integrated over all depths) during three autumn cruises. It is therefore necessary to establish a method for routine assessment of the grazing impact of pelagic tunicates which requires a minimum of effort in the field.

The gut pigment technique has been applied to appendicularians by a few authors (e.g. Jansa 1977, Gorsky et al. 1984, Landry et al. 1994), but its validity has not been established, nor have clearance and ingestion rates been calculated. In this study, I evaluate the gut pigment technique specifically for *Oikopleura vanhoeffeni*, a very abundant arctic and subarctic appendicularian (Udvardy 1954, Shiga 1993), using both laboratory experiments and wild-captured animals. The first of a set of three experiments

focuses on the comparison of the amount of chl *a* and an indigestible tracer ($^{68}$Germanium) within the gut. The use of chl *a* instead of the commonly used 'total pigment' circumvents problems usually associated with the use of the acid ratio equation in the presence of substantial amounts of chl *c* and *b* (Bianchi et al. 1995). In the second set of experiments, the gut passage times are determined by means of videotaping the paths of diatoms or corn starch particles through the guts of individual animals. In the third set of experiments, the possibility that enzymes contained in the phytoplankton food contribute to significant destruction of chl *a* during gut passage is explored. Chl *a* conversion factors and gut passage times are then applied to individuals of *O. vanhoeffeni* collected in the North Atlantic to estimate *in situ* clearance and ingestion rates.

### 3.2 Methods

Individual *Oikopleura vanhoeffeni* were collected in 450 ml wide-mouthed glass jars by SCUBA divers in Logy Bay, Newfoundland. Within minutes of each dive, the jars were put into a holding tank in the laboratory at +1 °C . Animals were either kept overnight, allowing them to reduce the particle concentration in the jars (experiments 1, 2 and 3), or used within a few hours of collection (experiment 4). Maintaining individuals in the original collecting jars has proven advantageous, since transferring them to other containers usually results in some mortalities. The first three experiments were performed during times of high particle concentrations in the water column (spring phytoplankton bloom), hence keeping the animals overnight ensured that most of the particles were

removed from the suspension and that the added diatoms (*Thalassiosira nordenskioldii*) contributed the largest portion of chl *a* in the jars (Table 3.1). In experiment 4, the ambient particle concentration was sufficiently low that addition of the laboratory diatoms after a few hours was enough to make *T. nordenskioldii* the dominant food source.

Preparation of the algae

One ml of $^{68}GeCl_2$ (New England Nuclear Corp.) was neutralized by adding equivalent amounts of 1N NaOH with a syringe through the rubber membrane covering the container, thus converting it into $^{68}Ge(OH)_4$ (unneutralized $^{68}GeCl_2$ is volatile, Azam and Chisholm 1973). Nine hundred µl distilled water were added to produce a working stock solution of 100 µCi $^{68}Ge(OH)_4$ $ml^{-1}$. *T. nordenskioldii* (Provasoli-Guillard, strain CCMP 997) was grown in 200 ml f/2+ medium (Guillard and Ryther, 1962) at 5 °C under continous illumination. During the mid-exponential phase, $^{68}Ge(OH)_4$ was added to the culture to produce a final concentration of 60-80 µCi $l^{-1}$ (Rivkin 1986). After 2 to 5 days, and just prior to performing the experiments, the entire culture was transferred to a reverse-flow filtration device with a 10 µm screen. Serial dilutions with GF/F filtered sea water removed most of the unincorporated label (< 3% of the original amount) and the culture was filled to the original volume of 200 ml. Eight 2-ml subsamples from this culture were filtered onto 1µm Nuclepore polycarbonate filters, four of which were put into 7-ml glass scintillation vials for determining $^{68}Ge$, and the remaining four into 1.5 -

ml Eppendorf centrifuge tubes for both pigment analysis and determination of $^{68}$Ge. Four 1-ml samples were taken and transferred into scintillation vials without filtration, and 3 to 5 subsamples were preserved in 2% glutaraldehyde for cell counts in a hemocytometer. These procedures yielded information on the amount of $^{68}$Ge and chl *a* per cell and the amount of $^{68}$Ge lost during the pigment extraction procedure (Table 3.2). The amount of $^{68}$Ge lost during procedures associated with the fluorometric determination of chl *a* was considerable, but very consistent within and between experiments (Table 3.2, 31-39 %). The loss of $^{68}$Ge may partially result from acidifying the samples during fluorometry, driving off $^{68}$Ge incorporated into the cell plasma, but not yet into the silica frustules. However, since all samples (water, cultures and animals) were treated identically, I assume that the losses were comparable, and no correction was applied.

Pigment conversion experiments with *O. vanhoeffeni*

Only feeding animals with intact houses were used for experimentation. Since the jars were filled to the brim, 50 ml of sea water was removed before 1 to 25 ml of culture was pipetted into the jars. The culture and water were gently mixed by moving the pipette slowly through the water while adding the culture and inverting the jars twice. All incubations were carried out in the dark for least 1.5 hours, corresponding to ca. two gut-passage times, but for no longer than 4 h. Each jar, containing one animal, was immersed in ice to keep the temperature between 0 to +1 °C. Following the incubations, 40 to 60 ml

of water were removed using a plastic syringe with a short attached silicon tube and filtered on 1 μm Nuclepore filters. The water was not mixed before samples were taken for two reasons: first, suspended particles were more representative of the food recently taken up by the animals, since the incubation time was much longer than the average gut passage time, and, secondly, fecal pellets produced by the animals during the incubation period would settle to the bottom and not contaminate the water samples. I checked for the presence of fecal pellets by examining the water during filtration. Most of the pellets remained trapped inside the gelatinous houses. Each 1μm Nuclepore filter was placed in a 1.5-ml Eppendorf centrifuge tube for pigment extraction. The pigment values and radioactivity of these water samples were used for calculating the pigment degradation for each individual animal, and thereby accounted for additional pigments derived from residual phytoplankton in the jars. The amount of chl *a* from phytoplankton other than the labeled diatoms was less than 25% of the total present and varied significantly within and between experiments (Table 3.1).

Immediately after each water sample was taken, the corresponding animal was processed. This was a very important step because disturbed animals stop feeding and usually cease producing the pharyngeal filter while digestion continues. Animals were removed via the escape chambers of their houses by means of a wide-mouthed pipette and the animals placed into 10 ml of ice-cold GF/F-filtered sea water in a petri dish. With the exception of experiment 2, animals were video recorded for 10 to 15 sec under a dissecting microscope with just enough white light to produce a clear image. The video

images were used to determine stomach contents and trunk lengths of animals. In experiment 2, I did not use the camera, and the trunk length was measured under dim red light to test whether photobleaching occurs during video recording under white light. Since this was not the case (Table 3.1), I proceeded to film the animals during the remaining experiments (experiments 3 and 4). Video images of the stomach contents gave me a third independent indicator of the amount of food in the gut in addition to pigments and $^{68}Ge$. Each animal was lifted out of its well plate with small forceps and held over a small Eppendorf centrifuge tube. Using micro-scissors, the trunk was cut off at the base of the tail and dropped into the centrifuge tube, thus ensuring that the trunk was not damaged before it reached the vial. Animals with crippled tails, but still alive, were incubated and used as blanks to correct for algae attached to the body surface. Subsequently, 750 µl or 1 ml of ice-cold 90% HPLC grade acetone was added to the centrifuge tubes. Both animals and filters were sonicated at a low setting using a Branson Sonifier (Cell Disruptor 200; setting 1. 4 pulses for less than 1 s each) and the samples were extracted overnight in a -20 °C freezer. Separate experiments showed that this level of sonication was sufficient to rupture the gut and dissolve its contents in acetone immediately, while not destroying any pigments.

The following day, the samples were agitated and then centrifuged at 10,000 rpm ( 9500 RCF, IEC Micromax centrifuge) for 3 min. Five hundred or 750 µl of the supernatant were diluted to 1.5 ml with 90% acetone, which is the minimum volume required in the cuvette, and measured in a Sequoia Turner fluorometer. Readings before

and after acidification with one drop 1N HCl were taken, although only the reading before acidification was used in subsequent calculations (see section on fluorometry). The entire procedure from handling of the animals to measurement of pigments in the fluorometer was performed under dim room light, just sufficient to allow for safe handling of the radiotoxic $^{68}$Ge. During all analytical stages, samples were kept on ice.

To determine the activity of $^{68}$Ge on the filters and in animals, pellets in the centrifuge vials were resuspended by returning some acetone from the fluorometer cuvette into the centrifuge tubes. The filters and pieces of animals were transferred from the centrifuge tubes into 7 ml scintillation vials. The centrifuge vials were rinsed 2 to 3 times using all the acetone from the cuvette, and the washings then added to the sample in the scintillation vials. This procedure minimized losses of $^{68}$Ge in the supernatant or on the wall of the centrifuge vials. The acetone was then evaporated at room temperature in a fumehood over a three day period (Penry and Frost 1991). Five ml of Ecolume scintillation cocktail (ICN) were added to the dried samples, which were then stored for at least 48 hours to permit $^{68}$Ge to come into transient equilibrium with $^{68}$Gallium (Rivkin 1986). The samples were counted with a window-setting of 0 to 1.9 MeV. Cpm was sufficient for calculation since only the relative amounts of $^{68}$Ge in the samples was needed. However, since $^{68}$Ge is a high energy emitter, the counting efficiencies are very high (85-95%, Rivkin 1986) and the cpm are almost equal to dpm. The Nuclepore filters had no quenching effect, as was determined by rerunning some animal samples in the scintillation counter after addition of Nuclepore filters. Since appendicularians ingest

whole cells, and 'sloppy feeding' as found in copepods does not exist, I assume that no particulate $^{68}Ge$ had been lost during the experiments (Tande and Slagstad 1985).

Field collections

On 2 and 24 May, and 9 June, 1995, animals were collected next to the Ocean Sciences Centre in Logy Bay (Newfoundland) by SCUBA diving and kept in a cooling bath at *in situ* temperatures for at least 2 h. This procedure ensured that the animals were undisturbed and continued feeding until they were removed from their jars. They were transferred into ice-cold filtered sea water to remove any attached algae and anesthetized with MS-222. After video recording at low light for 10 to 15 sec for later determination of trunk length, animals were frozen in liquid nitrogen and kept at -60 °C until analyzed. Three hundred ml of the water in the jar was filtered onto 1 µm Nuclepore filters and frozen in liquid nitrogen. For some animals, the houses in the jars were dissected to retrieve fecal pellets, which were pooled and frozen in liquid nitrogen.

Fluorometry:

For the pigment destruction experiments, a fluorometer was chosen for three reasons: 1) The gut pigment technique should be developed for *O. vanhoeffeni* in such a way that assessment of feeding rates is inexpensive, quick and simple. 2) Field data

processed by the same fluorometer already existed for *O. vanhoeffeni* from the North Water polynya (North East Greenland, Acuña et al., in prep.) and 3), no HPLC instrument was available for use with $^{68}$Ge. However, since the animals collected for the examination of natural gut pigment levels (1995) were measured in both the Sequoia-Turner fluorometer (model 450, narrow band NB440 excitation filter, and sharp cut SC665 emission filter) and the HPLC, the resulting chl *a* and pheopigment values could be compared in field samples. This comparison also showed that the standard acid-ratio equation yielded highly inflated pheopigment values in combination with the Sequoia Turner fluorometer equipped with the above mentioned filters (compare Axler and Owen 1994). These high pheopigment values may be the result of one of the two pheophytin *c* emission peaks shifting into the sensitivity range of the instrument after acidification, thereby increasing the total fluorescence after acidification. Therefore, instead of using the acid-ratio equation, I calibrated the Sequoia-Turner fluorometer with a *Thalassiosira nordenskioldii* culture using a spectrophotometer and the trichromatic equation according to Parsons et al. (1984). This procedure resulted in a good agreement between the Sequoia-Turner fluorometer and the HPLC methods for chl *a*, even in the presence of pheopigments or high amounts of chl *c* (Fig. 3.1). The advantage of using the diatom culture for calibration of the fluorometer was that the same culture was used for the pigment conversion experiments (for internal consistency) and that the pigment signature was very similar in the diatom dominated field samples (Fig. 3.2). As a result, I focus on chl *a* only and regard any chl *a* conversion into pheopigments or destruction into non-

fluorescent compounds as 'chl *a* conversion'. A more detailed description for the set-up and specifications of the reverse-phase HPLC can be found in Deibel et al. (in prep.).

Visual determination of the amount of food in the gut

The trunk lengths (Morris and Deibel 1993) and stomach contents of animals were measured directly from the video screen using calipers after the monitor screen was adjusted for any horizontal or vertical distortions. There are three compartments in the gut of *Oikopleura* in which the residence time of food is longer than in others: 1) the chamber near to the left lobe which contains the digestive gland. In this section, particles are uncompacted (Fenaux 1989). 2) the right lobe in which the food is packed into fecal pellets; and (3), the 'rectum', a compartment close to the anus which holds the fecal pellet before it is egested. For areas 2 and 3, the calculation of the volume of a prolate spheroid from a known length and width is a good approximation of food volume, since the food is already packed into the ovoid shape characteristic of oikopleurid fecal pellets. The first compartment, however, is flattened, so that application of this formula would result in an overestimation of the true volume. A rough estimate of the actual volume gave a factor of 1/3 of the volume of a prolate spheroid. I therefore continued to use the formula, but divided the calculated volume of the first compartment by three. Total food volume in the gut was simply the sum of food volume in all three compartments. In contrast, Deibel et al. (in prep.) calculated total food volume based on fecal pellets in the gut, not including

the first compartment, so the two studies are not strictly comparable in regard to food volume.

Calculations

Blanks for both pigment and $^{68}Ge$ analyses were subtracted from the original readings of filters and animals before final values were entered into formulae. Individuals with empty stomachs as determined from the video images, or with $^{68}Ge$ counts within 2 standard deviations of the blank counts, were excluded from the analyses. The amount of chl *a* present was determined by using a simple conversion factor from relative fluorescence to ng $ml^{-1}$ in the acetone extract. Since this study did not deal with pheopigments, any problems associated with calculating total pigments and molar conversions (Conover et al. 1986), as well as distinguishing different types of breakdown products (Head and Harris 1992), are avoided.

The chl *a* conversion efficiency (the amount of chl *a* changing into other fluorescent or non-fluorescent products) was calculated by the formula given by Penry and Frost (1991),

$$(1) \qquad d = \left(1 - \frac{cf / pf}{ca / pa}\right) * 100,$$

where d is the conversion of chl *a* in %, cf and ca are the cpm of $^{68}Ge$ in the food and animals, respectively, and pf and pa the amount of chl *a* in the food and animals, respectively.

An alternative way of showing the degree of chl *a* conversion is to regress the derived cell numbers from the chl *a* values against ingested cell numbers predicted from the conservative tracer (i.e. $^{68}Ge$) using the amounts of chl *a* and $^{68}Ge$ contained in the cultured cells (Table 3.2). However, since pigments other than the introduced radioactively-labeled diatoms were present in the jars, a correction factor (f) for this excess pigment had to be applied for each animal:

(2) $$f = (P_c * C_w)/(P_w * C_c),$$

where $P_c$ and $P_w$ are the pigment concentrations and $C_c$ and $C_w$ are the cpm of the culture and the experimental water, respectively. The resulting slope is equivalent to the proportion of intact chl *a* in the guts of the animals. Subtracting this value from one and multiplication by 100 gives another value for chl a conversion in %.

Gut passage times

Eight animals were fed with various concentrations of *T.nordenskioldii*. Since appendicularians are transparent, the passage of diatoms were video-recorded using a macro-lens. Nine additional animals were fed corn starch rather than diatoms. Corn starch is a highly visible marker and has previously been used for determining the gut passage times in salps by Madin and Cetta (1984) and in *O. vanhoeffeni* by Acuña et al. (in prep.). The gut passage time was defined from the point of impact of corn starch particles on the pharyngeal filter to defecation of the first fecal pellet containing white material. Following marker particles through the gut of an actively feeding animal seems to be more meaningful than determination of the 'gut clearance rate' as gut evacuation may be influenced by the amount of food in the gut (Kiørboe and Tiselius 1987 for copepods). In some animals, a 'bolus', similar to that described for salps by Harbison et al. (1986), formed when too high a corn starch concentration was used. Since these animals stopped ingesting material they were excluded from subsequent analysis.

Test of possible pigment degradation by enzymes or acids originating from the diatoms (*T. nordenskioldii*).

It has recently been hypothesized that enzymes originating from phytoplankton contribute to the breakdown of chl *a* in the guts of zooplankton (Spooner et al. 1994,

Head and Harris 1996). In particular, some diatoms such as *Skeletonema costatum*, *Phaeodactylum tricornutum* and *Nitzschia closterium* seem to contain high amounts of chl *a* degrading enzymes (Owens and Falkowski 1982). To test this hypothesis, ideally a proxy-gut would have to be devised which simulates the processes in the gut in the absence of digestive enzymes produced by the animal. However, too little is known about the gut of pelagic tunicates to simulate accurately *in vitro* the chemical processes involved. As an approximation, I assumed that the chemical properties of the guts were similar to those of sea water with the addition of cell contents of disrupted algae. The idea was to release enzymes and other substances from the algal cells and bring them into contact with chl *a*. To achieve this, a concentrated culture of *T. nordenskioldii* was sonicated for 10 - 15 sec to destroy all cells and release their contents (Branson Sonifier Cell Disruptor 200; setting 9, 10 to 15 s). This intensity of sonication was just enough to disintegrate the cells so that they were no longer visible under the microscope. The samples were incubated in darkness on ice (0 °C) for two hours, as in the experiments with *O. vanhoeffeni*. An incubation time of two hours was chosen, which was more than twice as long as the average residence time of the food in the gut of *O. vanhoeffeni*. Replicates of unsonicated algae served as controls. Following the incubation, 100 µl of sample volume were subsampled and diluted to 1 ml with 100% acetone. Subsequent pigment extractions and measurements were performed as described above.

## 3.3 Results

### Field collection

Comparisons of chl *a* in the field collected animals recorded by HPLC and Sequoia Turner fluorometer methods are shown in Figs. 3.1a - c. Large amounts of undegraded chl *a* and chl *c* were apparent, whereas only small amounts of pheopigments were present (Fig. 3.2). Average chl *a* values in animals from the first sampling date (2 May) were much higher than those in the other two collections, which was consistent with higher chl *a* levels in the water during the spring diatom bloom (Table 3.3). Typical HPLC profiles for pigments in the ambient water, field collected animals and fecal pellets, as well as the *T. nordenskioldii* culture used in the pigment conversion experiments, are shown in Fig. 3.2. Large amounts of chl *c* were found in the field collections, which is typical for diatoms (Fig. 3.2d, Table 3.3). The ratio of chl *a* to pheopigments was significantly lower during the spring bloom than during the other two sampling periods (Table 3.3, nonparametric 'Dunn's Method', Sigmastat; $p<0.05$).

### Chl *a* conversion

Fig. 3.3 shows a close agreement between chl *a* and the conservative tracer ($r^2$ = 0.80 and 0.91, for experiments 1-3 and experiment 4, respectively). Since $^{68}Ge$ cannot be

measured without error, slope and intercept parameters were calculated using geometric mean regression equations (model II, Sokal and Rohlf) in addition to the standard linear regressions (model I). The contribution of *T. nordenskioldii* to the total amount of chl *a* in the jars was high in all experiments, but varied significantly among the experiments (Table 3.1). There was no significant difference among the means of the different experiments, therefore an overall mean of pigment conversion could be calculated (i.e. 79%, Table 3.1). When the number of cells is calculated from chl *a* and $^{68}$Ge and values from the two techniques are plotted against each other (Fig. 3.4), the slope is an indication of how much the pigment technique would underestimate the true number of cells in the gut of the animals if not corrected for chl *a* conversion. The value of this slope is 0.24 (model II: 0.28, Fig. 3.4) which translates into a chl *a* conversion of 76% (model II: 72%), a value very close to the average chl *a* conversion of 79% calculated using formula 1 (Table 3.1). Chl *a* conversion was not significantly influenced by the size of the animals, nor by the amount of food in the ambient water (Table 3.4). The amount of food present in the animals determined by means of $^{68}$Ge and chl *a*, however, significantly influenced the chl *a* conversion (Table 3.4, Fig. 3.5), whereas this trend was not apparent in gut content volume determined from video analysis (Table 3.4).

Comparison of pigment, $^{68}$Ge and gut fullness

A comparison of the estimated amount of food in the gut from the video recordings with chl *a* and $^{68}$Ge is shown in Fig 3.6. Both chl *a* and $^{68}$Ge content of the animals are significantly correlated with the visually estimated gut volume ($p < 0.0001$ in both cases). The relationship between $^{68}$Ge and volume of food ($r^2 = 0.82$) is stronger than between chl *a* and volume of food ($r^2 = 0.52$).

Gut passage times

Table 3.5 summarizes the results of gut passage time experiments with diatoms and corn starch. The size of animals in the range from 2.0 to 4.8 mm trunk length did not have a significant effect on the gut passage time. Although some mixing may occur in the first compartment located near the left lobe, the effects are negligible, since the front edge of the marker is clearly visible once the fecal pellet has been formed. Typically, the first compartment of a continuously feeding animal is always filled with food while the remaining sections of the gut contain one or two compacted pellets. The overall gut passage time was 0.8 h (Table 3.5).

Test of possible degradation of chl *a* by enzymes originating from diatoms used in chl *a* conversion experiments (*T. nordenskioldii*)

During the 2 h incubation of the cell homogenate, there was no sign of chl *a* conversion into pheopigments or non-fluorescent products (Table 3.6). This indicates that enzymes from the algae did not contribute to the observed chlorophyll conversion in the experiments with *O. vanhoeffeni*. However, sonication destroyed about 50 % of chl *a*, as shown by the comparison with the intact cells (Table 3.6). Note that the level of sonication used to disrupt the animals was much lower (setting 1, 4 pulses for less than 1 s each) than the level used to destroy all diatom cells (setting 9, 10 to 15 s). As mentioned above, the low level of sonication had no effect on the pigment destruction, which was tested in preliminary experiments (unpubl.)

## 3.4 Discussion

Using chl *a* rather than total pigment

The acid ratio equation yielded inflated values for pheopigments from the Sequoia-Turner fluorometer equipped with an NB 440 (440 ± 5 nm) excitation filter when compared to HPLC (data not shown). These inflated values are probably due to one of the pheophytin *c* peaks shifting into the sensitivity range of the fluorometer after

acidification. This may have an effect on the reading, although the fluorescence of pheophytin *c* is known to be only about 16% of the original chl *c* fluorescence (Holm-Hansen et al. 1965). However, when fitted with the narrow-band excitation filter, the instrument is selective for undegraded chlorophylls and discriminates against *a* -type pheopigments, which therefore renders the acidification procedure unnecessary. Calibrated with a *T. nordenskioldii* culture rather than pure chl *a*, the setting was corrected for residual fluorescence from other pigments in the range sensitive for chl *a*. This procedure gave the best agreement with HPLC values (Fig. 3.1) and avoided most of the problems identified earlier by Bianchi et al. (1995) when comparing HPLC with fluorometry. Yet using a narrow band filter for the emission as well as for the excitation, as suggested in Welshmeyer (1994), would probably improve the method described above.

The advantages of using $^{68}$Ge as a conservative tracer

Preliminary tests using biogenic silica to determine the extent of pigment destruction (Conover et al. 1986, Head and Harris 1996) showed that single animals or small samples of fecal pellets are below the detection limit of this method (unpubl.). However, reliable chl *a* values could still be obtained using the same amount of material. By using the biogenic silica technique, one has to rely on a relatively crude method as a standard for the much more sensitive fluorometric technique. Small errors in the many

analytical steps of the biogenic silica method, such as the hydrolysis, can accumulate. In order to obtain an adequate signal, animals would have to be pooled. An important limitation for this study (as is typical for work with gelatinous zooplankton) was the scarcity of healthy animals, and pooling would have drastically reduced the number of possible replicates. The second problem with pooling is the loss of information on variation between individuals. The use of $^{68}$Ge instead of biogenic silica, as suggested by Penry and Frost (1991), circumvents most of these problems, but the $^{68}$Ge method shares the one major disadvantage of the silica method in that it can only be used where diatoms or silicoflagellates are ingested. For both biogenic silica and $^{68}$Ge, the assumptions of conservative tracers must hold true to be useful for the ratio method (Tande and Slagstad 1985). I have tested $^{68}$Ge as a conservative tracer in chapter 4, and found that only negligible amounts of $^{68}$Ge were absorbed by the animals.

General considerations concerning pigment destruction in zooplankton

The assumption that all chlorophyll *a* is stoichiometrically converted into fluorescent pheopigments in the guts of zooplankton (Shuman and Lorenzen, 1975) has been refuted by evidence showing degradation of chlorophyll into nonfluorescent compounds (Helling and Baars 1985, Wang and Conover 1986, Head 1988). This evidence includes observations on copepods in which Head and Harris (1996) found that most of the chlorophylls degrade into nonfluorescent products rather than into pheopigments and that pheopigments do not seem to be intermediates. These findings

strengthen the approach I chose to determine chl *a* conversion rather than 'destruction of total pigment'. Rather than dealing with a wide array of fluorescent by-products and different conversion velocities, I focused on one pigment only (i.e. chl *a*) and regarded any type of alteration of its original structure except the loss of the phytol chain as 'chl *a* conversion'. Chlorophyllide *a* has the same fluorescent characteristics as chl *a* and would therefore be indistinguishable from chl *a* with a fluorometer (Lorenzen and Downs 1985). I hope this method will also accommodate a wider range of food types, since the chl *a* molecule is universal whereas degradation pathways and therefore breakdown products may vary among phytoplankton species. The problem of 'true weights' and 'chl *a* equivalent weights' used for pheopigments, as pointed out by Conover *et al.* (1986), is completely avoided by using chl *a* only.

Using animals instead of feces to calculate pigment degradation

In many cases, the calculation of pigment destruction has been based on the comparison between food and feces, and not between food and animals (Conover et al. 1986, Penry and Frost 1991, ). This is in spite of the fact that, per definition, the gut pigment technique measures the fluorescence in the animals and not in the feces (Mackas and Bohrer 1976). The examination of animals produces "snapshot" data with a certain amount of undigested pigments in the guts, whereas fecal pellets contain the completely digested fraction (Penry and Frost 1991). In experiments by Conover et al. (1986), fecal

pellets remained from 12 to 48 h in the experimental containers before they were analyzed. This may have lead to further degradation of chl *a*, although according to Head and Harris (1996), most of the pigment degradation occurs during an early stage of ingestion and only a small amount is lost in the feces. It would indeed be beneficial for the application of the gut pigment technique if there were no further degradation after an initial and sudden decrease of pigment levels during ingestion, as suggested by Head and Harris (1996), since it would make the samples less susceptible to changes in handling and processing times. However, unless it is demonstrated that extracellular enzymes or even bacteria do not further degrade chl *a* after this initial decrease, it is safer to compare directly pigment and conservative tracers in the animals rather than the feces to ensure that the appropriate correction factors are applied. The protocol for pigment degradation experiments should therefore be as close as possible to the collection protocol of field studies. In my experiments, the only difference between the pigment degradation experiments and the field collection of *O. vanhoeffeni* in the Labrador Current was two additional steps in the analysis of the field collected animals: they were (1) frozen in liquid nitrogen and (2) stored at -60 °C for several months, assuming no pigment losses during this time (Redden et al. 1993). The protocols for sampling, extraction and fluorometry were identical.

Chl *a* conversion in *O. vanhoeffeni*

By including data from the cultures (i.e. cell numbers per volume, chl *a* and cpm per cell, Table 3.2), one can calculate the number of cells in the guts of the animals from the known amounts of $^{68}$Ge and chl *a* in the animals. The slope between cell number estimates from $^{68}$Ge and that from chl *a* is another indicator of the degree of pigment conversion (Fig. 3.4). The value of the slope in Fig. 3.4 is equivalent to the amount of intact chl *a* (i.e. 1-chl *a* conversion efficiency). This procedure may cause problems with residuals in the lower range of gut content since the regression line is rotated around the mean by values at the 'high end' (Fig. 3.4). However, in this case the mean conversion efficiency calculated from individual conversion efficiencies (Table 3.1) and the conversion efficiency calculated from the slope (Fig. 3.4) were similar (i.e. 79 and 76%, respectively), supporting the overall linearity of the data set.

The pigment profiles for animals used in the experiments, as well as those collected in Logy Bay, were characterized by dominant chl *a* and chl *c* peaks (Table 3.3, Fig. 3.2). The conversion of chl *a* was consistent among the different sampling days (Table 3.1). This is a somewhat surprising result, since the animals had very different feeding histories, spanning the decline of the spring diatom bloom to the lower biomass concentration typical of the summer (Table 3.3). In contrast, variable and unknown

feeding histories have been considered a major problem in establishing pigment destruction factors for copepods (Penry and Frost 1991).

Considering the average chl *a* conversion efficiency of 79 % and the small amounts of pheopigments recovered in field collected animals (< 20 %, Table 3.3), pheopigments cannot account for all the chl *a* breakdown products. This suggests that the majority of chl *a* is degraded into non-fluorescent products rather than pheopigments. Chl *a* degradation into non-fluorescent products has also been observed by Madin and Purcell (1992), who found that estimates based on total pigments (chl *a* and pheopigments combined) were 2.2 to 2.5 times lower than estimates based on counts of diatom frustules in the feces of *Cyclosalpa bakeri*. The question of whether pheopigments are produced by separate pathways (Head and Harris 1996) or whether they are part of the chl *a* degradation process cannot be answered from the present data. For pelagic tunicates in general, the high degree of pigment destruction into non-fluorescent products, as shown for salps (Madin and Purcell 1992) and appendicularians (this study), imposes problems for studies which have so far ignored this issue (e.g. Drits et al. 1992), and actual ingestion rates may be higher than estimated by these authors.

The dependence of chl *a* conversion on the amount of food in the water and the gut

The pigment conversion efficiency was inversely related to $^{68}$Ge and chl *a* in the guts of the animals (Table 3.4, Fig. 3.5). However, the observed relationship between conversion efficiency and chl *a* or $^{68}$Ge in the animals could be partly attributable to an autocorrelation of the variables, since both chl *a* and $^{68}$Ge appear in the calculations of the chl *a* conversions (Krambeck 1995). When the conversion efficiencies were plotted against total volume of food in the gut, the inverse relationship disappeared (Table 3.4). However, if an autocorrelation alone were responsible for the observed pattern, the slopes of the regression lines would have opposite signs because $^{68}$Ge appears in the numerators and chl *a* in the denominators of equation 1. Both regressions, however, show a similar negative trend. In addition to the constant conversion efficiency of 79%, I will therefore use the regression equation in Fig. 3.5 as an alternative way of correcting for chl *a* conversion. I will then calculate clearance rates using both the constant and this new 'variable chl *a* conversion' (see below).

Gut passage times in *O. vanhoeffeni*

Mean gut passage times were found to be nearly identical for both diatoms and corn starch particles (Table 3.5), showing that corn starch is a useful marker for gut passage time experiments in appendicularians as well as in salps (Madin and Cetta 1984).

I found no evidence that gut passage time changes with animal size (Table 3.5). However, the power of the regression was low (i.e. $1-\beta = 0.088$) due to the low *effect size*[1] ($r = 0.167$) and the low number of replicates ($n = 16$) (Cohen 1969). Therefore, it may not be possible to exclude a small influence of size on gut passage time (the effect would range from 42.4 min at 2 mm, to 49.8 min at 5 mm), but this difference would be biologically insignificant considering other sources of variance and the shallow slope. Furthermore, the size range of animals (1.8 - 5 mm) in the laboratory observation of gut passage was similar to the range of field-collected animals (2.9-5 mm), so that the calculated overall mean should be applicable. For comparison, body size influenced gut passage time in the salps *Pegea confoederata* and *Salpa maxima*, but not in *Salpa cylindrica* (Madin and Cetta 1984).

Separate observations of fecal pellet production rates have revealed that the time interval between production of individual fecal pellets remained remarkably constant over wide food concentration ranges for individual animals, which strongly indicates that food concentration has no effect on the observed gut passage times (chapter 1). I therefore used a single value of 0.8 h for gut passage time. The observed variability in gut passage time is due to variation between individuals (chapter 1), but is also inherent in the method: although *O. vanhoeffeni* performs as a conveyor-belt feeder and essentially no mixing occurs during gut passage, food retention time varies in different compartments of the gut.

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[1] 'effect size' is a term used in the statistical literature and should not be confused with the effect of size discussed in this paragraph.

As long as the pharyngeal filter is being produced, food is continuously transported into the first compartment. However, food remains there until a certain amount (equivalent to the content of one fecal pellet) 'spills' into the second compartment, where a fecal pellet is formed. Thus if a marker particle is ingested together with food just prior to the 'food pulse', the marker can be packed into a pellet very quickly and the measured gut passage time is short. If the marker particle is ingested just after the 'pulse', the measured gut passage time is correspondingly longer. Since the average time interval between fecal pellets is ca. 16 min (chapter 1) this factor alone introduces considerable variability even within one individual.

Chlorophyll bleaching enzymes derived from the diatoms

There were no significant changes of chl *a* during 2h incubations of the cell homogenate (Table 3.6). However, there was an initial loss of chl *a* after sonication, caused either by the sonication procedure itself (Spooner et al. 1994) or by the release of substances (such as acids from the vacuoles) which may degrade chl *a* (Table 3.6). There was no evidence for chlorophyllase activity, since chl *a* levels in the cell homogenate remained as stable as those in undamaged cells for 2 h, a period of time which was more than twice as long as the average gut passage time (Table 3.6). Head and Harris (1996) have suggested that 'chlorophyll bleaching enzymes' present in the food are responsible for most of the observed pigment destruction, although their data did not rule out the

possibility that the enzymes responsible for pigment destruction are entirely produced by the animals (Bochdansky and Deibel 1997). In some studies in which high levels of chlorophyllase were measured (Owens and Falkowski 1982), experiments were performed in acetone extracts. In contrast, no chlorophyllase activity could be detected in 80% methanol extracts of the same algae (*Nitzschia*, *Phaeodactylum* and *Skeletonema*) by Moreth and Yentsch (1970), who concluded that chlorophyllase seems most active in 50% acetone. Therefore, clarification is necessary regarding the level of chlorophyllase activity in the aqueous solution present in an animal gut at *in situ* temperatures and for a time equal to the gut residence time. One such piece of evidence has been presented by Spooner et al. (1994) for *Pheodactylum* in experiments similar to ours using *Thalassiosira nordenskioldii*. The authors concluded that a significant reduction (defunctionalisation) of chl *a* can occur in algal homogenates at time scales similar to the gut passage times of zooplankton. The experiments with *T. nordenskioldii*, however, did not show chlorophyllase activity in the cell homogenates, but strong degradation of chlorophyll in the guts of the animals. I suggest three possible explanations for the discrepancies between my experiment and that of Spooner et al. (1994). Firstly, as Owens and Falkowski (1982) pointed out, algae from the genus *Thalassiosira* display much lower chlorophyllase activity than does *Phaeodactylum*. Therefore, future pigment degradation experiments need to be designed to test whether food such as *Phaeodactylum* significantly changes the pattern of pigment destruction which I have observed using *Thalassiosira nordenskioldii*. Secondly, experimental temperatures (ca. 0 °C in this study as opposed to 20 °C in Spooner et al. 1994) could also influence the fate of pigments. Thirdly, most of

the chl *a* in the experiments by Spooner et al. (1994) was converted into chlorophyllide, which would not be distinguishable from chl *a* with a fluorometer. Pyropheophorbides, commonly found in copepods (Head and Harris 1992) and to a lesser extent in *O. vanhoeffeni* (Fig. 3.2b), did not appear until 3 h after the start of incubation, and only trace amounts were produced (< 0.1 % of the total amount of chloropigments, Spooner et al. 1994). Furthermore, HPLC profiles of field collected *O. vanhoeffeni* (Fig. 3.2b) and copepods (Head and Harris 1992, < 0.5 % of total pigments) show very little chlorophyllide in the guts of grazers. The pigment composition resulting from algal chlorophyllase activity in Spooner et al. (1994) is therefore very different from a typical profile from herbivorous grazers or their fecal pellets. Therefore, the data of Spooner et al. (1994) indicate that algal chlorophyllase has little influence on the breakdown of chl *a* in the guts of zooplankton and that another pool of enzymes, such as the digestive enzymes of the animals, converts most of the chl *a* into either pyropheophorbide or nonfluorescent breakdown products.

Application to the field

The agreement between chl *a* and $^{68}$Ge data from laboratory experiments strongly suggests that chl *a* can be used to estimate ingestion rates after correction for pigment conversion (Fig. 3.4). As the laboratory experiments showed, there was also good agreement between food volume in the gut and $^{68}$Ge content, and to a lesser extent

between food volume and chl *a* content (Fig. 3.6). It is more difficult to delineate the food patch in the first compartment where the food is not yet compacted. The first compartment would also contain the largest amount of undegraded chl *a* since it continuously receives fresh material from the esophagus. In combination, these two factors would increase the variance around the regression line shown in Fig. 3.6b and are a likely cause of the lower $r^2$ compared with the $r^2$ of the regression between $^{68}$Ge and gut volume (Fig. 3.6a).

Although the amount of chl *a* conversion was consistent between experiments with *T. nordenskioldii*, it is unclear if chl *a* conversion is similar in animals feeding on natural food mixtures. In order to test whether chl *a* conversion and gut passage times I determined are representative for animals in the field, I will use the laboratory data in conjunction with the gut pigment content of the field collected animals and the chl *a* in the water in the collecting jars to calculate clearance rates and compare these with existing data of clearance rates of *O. vanhoeffeni* (Deibel 1988, Knoechel and Steel-Flynn 1989, Morris and Deibel 1993). In Deibel (1988), the number of latex beads retrieved from both animals and houses have been used to calculate clearance rates of freshly caught animals in natural sea water. In Morris and Deibel (1993), a model of water flow through the house was presented which took morphological and behavioral variables into account. When the examined particles are retained with 100% efficiency, the flow through the tail chamber equals the clearance rate (Morris and Deibel 1993). In Knoechel and Steel-Flynn (1989), *O. vanhoeffeni* was fed $^{14}$C-labeled diatoms in an apparatus

specifically designed for measuring clearance rates *in situ*. The three resulting allometric relationships for the different methods for estimating clearance rate are shown in Fig. 3.7 and compared with three estimates of clearance rates derived from the gut pigment technique developed in this study. Clearance rates were estimated using (1) uncorrected pigment values, (2) a constant correction factor of 4.76 (i.e. 79% chl *a* conversion, Table 3.1) and (3) 'variable conversion factors'. These variable conversion factors were derived by using the regression equation in Fig. 3.5. As Fig. 3.7 shows, the values derived from the variable conversion factor were higher than those obtained using the constant multiplication factor of 4.76, since the field animals had on average less chl *a* in the gut than did those in the pigment conversion experiments. However, a problem of using this variable correction factor is animal size. With this procedure, smaller animals automatically receive higher conversion efficiency factors than larger animals.

The highest estimates for clearance rates of *O. vanhoeffeni* in Fig. 3.7 come from the ideal model (Morris and Deibel 1993), which assumes 100 % retention efficiency, 50% time spent feeding, an unclogged filter surface and a pharyngeal filter which is in place during the entire time period; in other words a physiological maximum performance. The clearance rates calculated on the basis of the uncorrected chl *a* form the lower envelope. The data from the study using latex beads in field-collected animals (Deibel 1988) are very close to the estimates using variable conversion factors for each body size class. The mean clearance rates calculated by Knoechel and Steel-Flynn (1989) fall within the 95% confidence limits of the estimates based on the constant conversion

factor for three out of five body size classes (Fig. 3.7). Given the wide range of variability among individual animals and the problems associated with all experimental techniques, including the handling of these delicate organisms, the overall means are within a threefold range, except for the uncorrected pigment data. Since the dependence of the magnitude of chl *a* conversion on the amount of food in the gut is questionable, and animal size has not been incorporated into the model, using the constant correction factor is probably the most conservative approach. An important factor in comparing various methods is whether the clearance rate estimates incorporate particles trapped in the house or only those ingested by the animal (chapter 1 and 2). Gorsky et al. (1984) estimated that 30% of particles cleared from suspension were not ingested. One would therefore expect clearance rates based on the gut pigment technique to be lower than values from other methods (i.e. Deibel 1988, Knoechel and Steel-Flynn 1989, Morris and Deibel 1993), but not as low as estimates using uncorrected pigment values (Fig. 3.7).

Madin and Kremer (1995) also conclude that there may be an "irreducible minimum" variability from one individual salp to another, but that the gut pigment technique gives reasonable results despite its uncertainties. Overall, the clearance rate values based on the gut pigment technique lie well within the ranges given by alternative methods. Considering its simplicity, the gut pigment technique has great potential for routine *in situ* determination of feeding rates in pelagic tunicates.

## 3.5 Conclusions

Despite the wide application of the gut pigment technique in zooplankton ecology, this is the first evaluation of its usefulness for appendicularians. The cold water appendicularian, *Oikopleura vanhoeffeni*, is large enough for individual animals containing significant amounts of undegraded chl *a* to be used in fluorescence analysis. The technique was improved by using an excitation filter in a fluorometer which was selective for chlorophylls and discriminated against *a*-type pheopigments. This novel approach reduced problems associated with the use of fluorometers in studies of gut fluorescence. The comparison with the conservative tracer $^{68}$Ge showed that most of the chl *a* was converted into undetectable non-fluorescent pigments and not into pheopigments. The amount of chl *a* conversion using diatoms as food ranged from 61 to 88%, depending on the amount of food in the gut, and was not significantly affected by body size. Separate incubation of sonicated cell extracts suggested that digestive enzymes derived from the animals rather than chlorophyllase from the diatoms were responsible for the majority of chl *a* conversion observed in the laboratory. The gut passage times were unrelated to food concentration and trunk length with an overall mean value of 0.8 h. Applying these laboratory estimates of chl *a* conversion and gut passage time to field-collected animals, the estimated clearance rates fell well within the range of published results from alternative methods.

### 3.6 Tables

Table 3.1: Chl *a* conversion into pheopigments and nonfluorescent breakdown products in *Oikopleura vanhoeffeni* feeding on *Thalassiosira nordenskioldii*. Means of different experiments were not significant (pairwise GT2 test at $\alpha = 0.05$; and GLM for the class variable 'experiment', $p = 0.25$, $n = 49$). '% additional chl *a*' indicates the amount of chl *a* in the jars which did not derive from added *T.nordenskioldii*. Since there was no significant difference among the experiments the data were pooled and an overall mean was calculated.

| experiment # | date (1995) | number of animals | % degradation | 95% confidence limits | % additional chl *a* |
|---|---|---|---|---|---|
| 1 | 9 May | 12 | 75 | 71 - 80 | 7 |
| 2 | 22 May | 11 | 83 | 74 - 92 | 25 |
| 3 | 3 June | 5 | 86 | 78 - 94 | 6 |
| 4 | 7 June | 21 | 78 | 71 - 84 | 14 |
| overall: | | 49 | 79 | 76 - 83 | 14 |

Table 3.2: Activity of incorporated $^{68}$Ge label and chl *a* content in cells of *Thalassiosira nordenskioldii* cultures used in chl *a* conversion experiments with *Oikopleura vanhoeffeni*. '% loss of $^{68}$Ge label' refers to the percentage of $^{68}$Ge activity lost from the cells during the pigment extraction procedure.

| experiment | n | cpm cell$^{-1}$ ± SD | chl *a* (pg cell$^{-1}$) ± SD | % loss of $^{68}$Ge |
|---|---|---|---|---|
| 1 | 4 | 0.46 ± 0.023 | 1.74 ± 0.07 | 36 |
| 2 | 4 | 0.44 ± 0.016 | 1.47 ± 0.07 | 31 |
| 3 | 4 | 0.42 ± 0.035 | 1.46 ± 0.14 | 39 |
| 4 | 4 | 0.37 ± 0.006 | 1.89 ± 0.03 | 38 |

Table 3.3. Chl *a* content and pigment ratios of the water and individual *Oikopleura vanhoeffeni* during May - June 1995. The chl *a* values are based on measurements with the Sequoia-Turner fluorometer. The ratios of chl *a* and chl *c*, and chl *a* and *a*-type pheopigments (chl *a* : *a* pheo) were measured using HPLC analysis. nd = not determined.

| date 1995 | chl *a* ± SD (n) ($\mu g\ l^{-1}$ or $ng\ ind^{-1}$) | chl *a* : chl *c* ± SD (n) | chl *a* : *a* pheo ± SD (n) |
|---|---|---|---|
| water | | | |
| 2 May | 1.35 ± 0.49 (15) | 1.75 ± 0.04 (4) | 24.5 ± 1.8 (4) |
| 24 May | 0.14 ± 0.05 (17)( | nd | nd |
| 9 June | 0.35 ± 0.13 (15) | nd | nd |
| animals | | | |
| 2 May | 18.92 ± 18.28 (12) | 1.11 ± 0.030 (11) | 5.7 ± 6.9 (11) |
| 24 May | 2.36 ± 1.37 (14) | 2.27 ± 1.72 (12) | 33.4 ± 29.5 (11) |
| 9 June | 2.17 ± 1.02 (10) | 1.57 ± 0.69 (10) | 57.6 ± 47.1 (5) |

Table 3.4. Exploratory linear regression analyses of variables in relation to chl *a* conversion (dependent variable) in laboratory experiments. Since the two outliers in (5) have not been removed, values are different from those given in Fig. 3.5. '*' highlights p-values <0.05.

| (1) variables (units) | mean | range | $r^2$ | n | p |
|---|---|---|---|---|---|
| (2) trunk length (mm) | 3.53 | 1.79 - 4.96 | 0.002 | 48 | 0.79 |
| (3) chl *a* conc in the water ($\mu g\ l^{-1}$) | 2.53 | 0.08 - 15 | 0.026 | 49 | 0.27 |
| (4) $^{68}Ge$ conc in the water (cpm $ml^{-1}$) | 323 | 6 - 2207 | 0.018 | 49 | 0.36 |
| (5) chl *a* in the animals (ng $ind^{-1}$)** | 30 | 0.08 - 269 | 0.25 | 49 | 0.0002 * |
| (6) $^{68}Ge$ in the animals (cpm $ind^{-1}$) | 13876 | 176 - 83276 | 0.12 | 49 | 0.0077 * |
| (7) total volume of food in the gut from video ($mm^3\ ind^{-1}$) | 0.099 | 0.004 - 0.33 | 0.004 | 36 | 0.73 |

Table 3.5: Gut passage time of *Oikopleura vanhoeffeni*. In 1994, *Thalassiosira nordenskioldii* cells were followed through the gut, using video recording, from impact on the pharyngeal filter to defecation of the fecal pellet. In spring 1995, corn starch was used in addition to algae. Both methods gave essentially the same result, therefore a grand mean was calculated. The gut passage time was independent of trunk length in the range of animals considered ($r^2$=0.028 ,p>>0.05 , n=16). One trunk length measurement was missing.

| date | marker particles | temp (°C) | n | mean gut passage time (h) | ± SD | mean trunk length (mm) |
|---|---|---|---|---|---|---|
| June 1994 | *T. nordenskioldii* | +2 to +6 | 8 | 0.82 | 0.19 | 3.9 |
| June 1995 | corn starch | +1 | 9 | 0.77 | 0.19 | 3.7 |
| overall | | | 17 | 0.79 | 0.18 | 3.8 |

Table 3.6: Chl *a* degradation in a cell homogenate of *Thalassiosira nordenskioldii*. Cells were destroyed by sonicating the culture on ice for 10 sec. The homogenate was then incubated on ice in the dark for 2 h (i.e. > 2 gut passage times), similar to the chl *a* conversion experiments with *Oikopleura vanhoeffeni*. Three controls were necessary to show whether or not enzymes derived from the diatoms destroy chl *a*: 1) Cells incubated for 2 h without sonication (intact cells), 2) cells immediately frozen in liquid nitrogen without sonication (intact cells), and 3) cells sonicated and immediately frozen. Means with the same letter are not significantly different at the $\alpha$=0.05 level according to a Tukey's studentized range (HSD) test (SAS statistical software).

| | n | mean chl *a* (ng $ml^{-1}$ ± SD) | |
|---|---|---|---|
| <u>treatment:</u> | | | |
| sonicated & incubated | 6 | 42.1 (± 15.36) | A |
| <u>controls:</u> | | | |
| incubated (intact cells) (1) | 6 | 67.7 (± 3.57) | B |
| immediately frozen (2) | 6 | 72.4 (± 11.23) | B |
| sonicated & immediately frozen (3) | 6 | 28.1 (± 16.72) | A |

## 3.7 Figures

Fig. 3.1. Comparison of chl *a* measurements using the Sequoia-Turner fluorometer (calibrated with a *Thalassiosira nordenskioldii* culture) and HPLC methods in field-collected animals from Logy Bay during three periods in spring 1995. The solid line represents the linear regression through the data points and the dashed line the ideal case of a 1:1 relationship between the two variables. The slope of the regression line is significantly different from 1 in a) ($y = -0.54 + 0.81x$, $r^2 = 0.91$, $n = 12$, $p = 0.032$), mainly because of the point on the far right of the x-axis (> 70 ng $ind^{-1}$). The slopes in b) and c) are not significantly different from 1 (for b: $y = 0.60 + 0.87\ x$, $r^2 = 0.63$, $n = 14$, $p = 0.51$; for c: $y = -0.43 + 1.23\ x$, $r^2 = 0.87$, $n = 10$, $p = 0.20$).

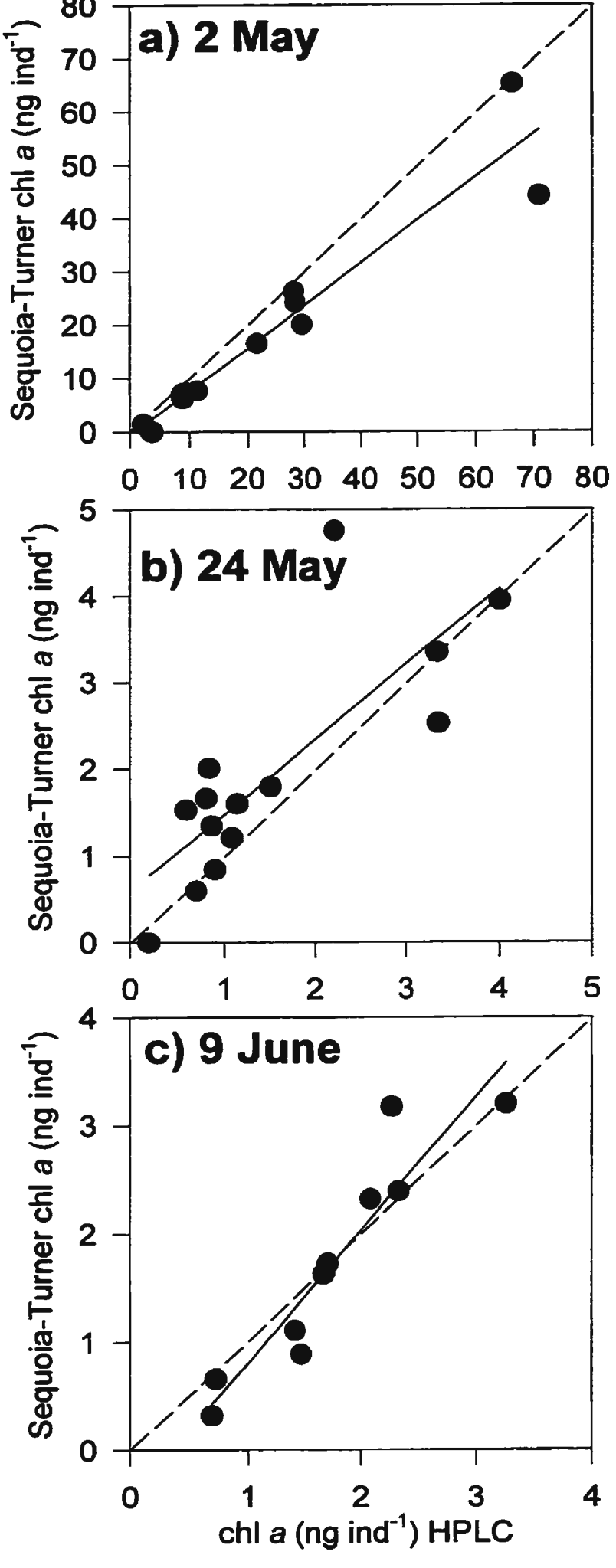

a) 2 May
b) 24 May
c) 9 June
Sequoia-Turner chl a (ng ind-1)
chl a (ng ind-1) HPLC
0
10
20
30
40
50
60
70
80
1
2
3
4
5

Fig. 3.2. Typical HPLC profiles of water samples (a), animals (b), feces (c) and the diatom culture (d, *Thalassiosira nordenskioldii*) used in the chl *a* conversion experiments. 1 - chlorophyllide *a*, 2 - chl *c*, 3 - pheophorbide *a*1, 5 - pyropheophorbide *a*, 9 - chl *b*, 10 - chl *a* allomers, 11 - chl *a*, 15 pheophytin *a*, 17 - pyropheophytin *a*.

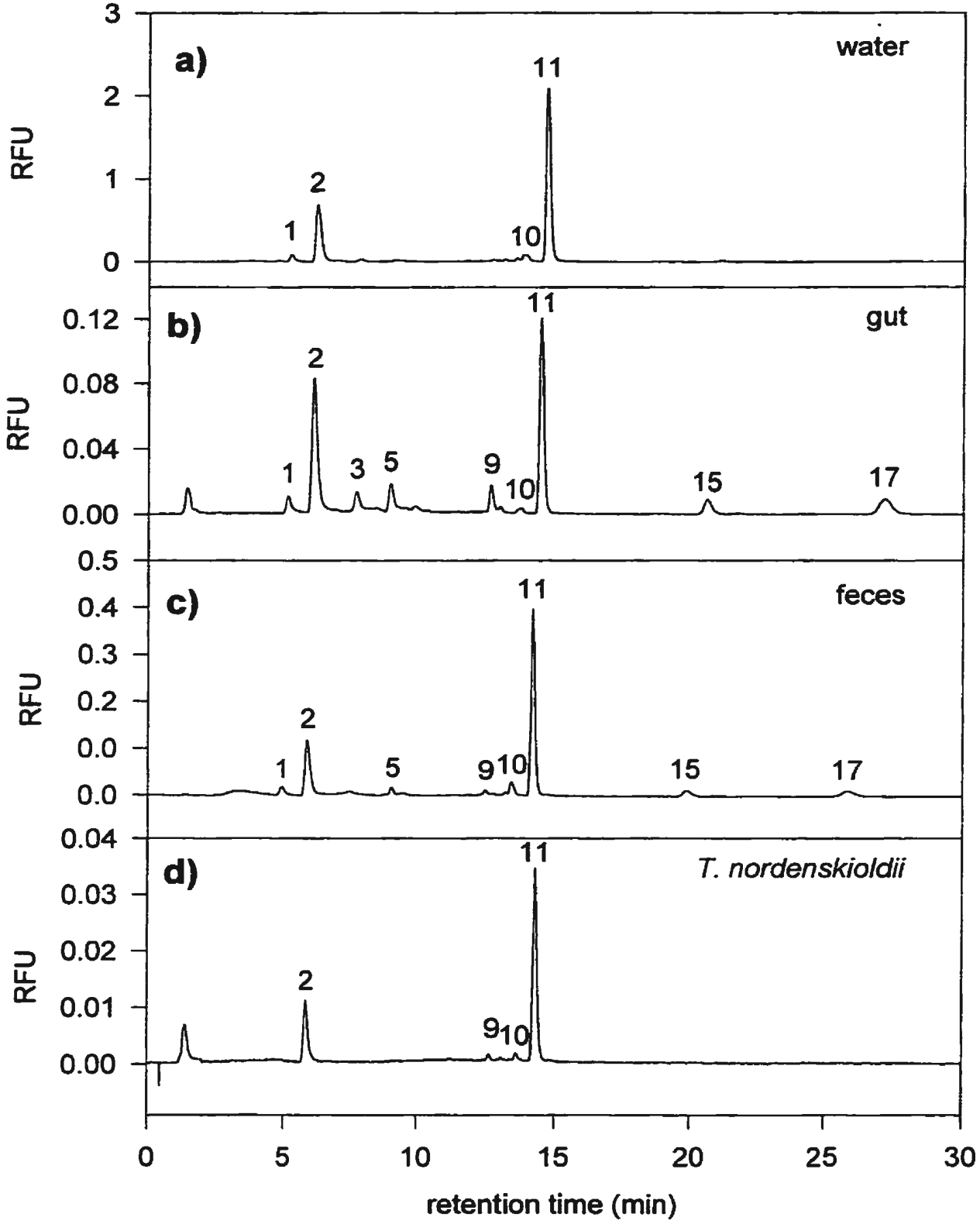

a)
water
b)
gut
c)
feces
d)
T. nordenskioldii
RFU
retention time (min)
1
2
3
5
9
10
11
15
17

Fig. 3.3. Relationship between chl *a* measured using the Sequoia-Turner fluorometer and the conservative tracer $^{68}Ge$ in the guts of experimental animals. For Fig. 3.3a experiments 1 to 3 have been pooled since the cultures displayed similar labelling pattern and chl *a* contents per cell (Table 3.2). The datum point in brackets was excluded from the regression analysis, since it would have had extreme weight in calculating the slope.

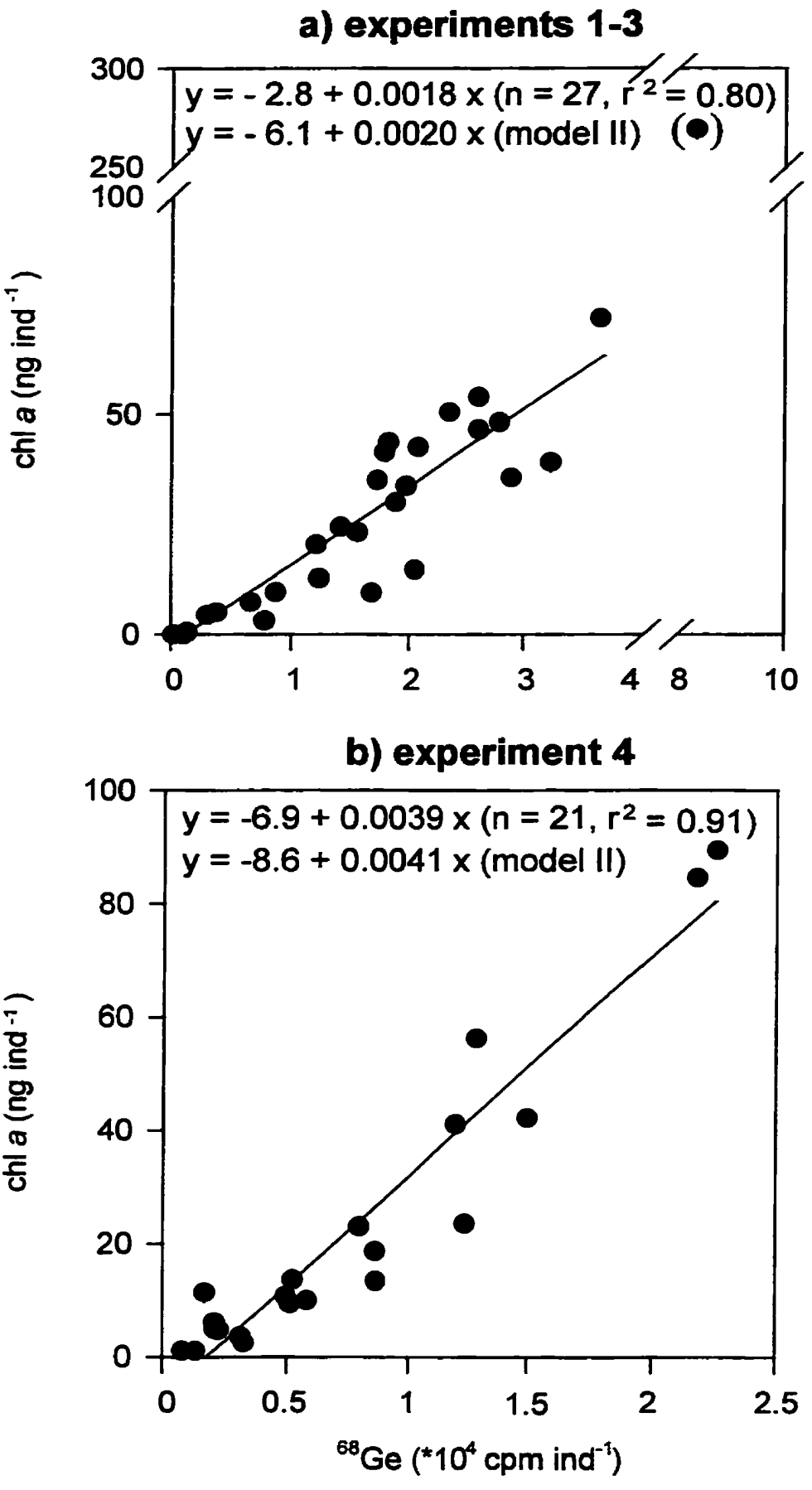

a) experiments 1-3
y = - 2.8 + 0.0018 x (n = 27, r 2 = 0.80)
y = - 6.1 + 0.0020 x (model II) (●)
chl a (ng ind-1)
300
250
100
50
0
0
1
2
3
4
8
10
b) experiment 4
y = -6.9 + 0.0039 x (n = 21, r2 = 0.91)
y = -8.6 + 0.0041 x (model II)
chl a (ng ind-1)
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
68Ge (*10^4 cpm ind-1)

Fig. 3.4. Comparison of the number of cells estimated in the guts of individual animals calculated by two different approaches: on the basis of the measured amounts of chl *a* and $^{68}$Ge in the animals, and a known amount of chl *a* and $^{68}$Ge per cell. The slope of the linear regression is another indicator of the overall amount of chl *a* conversion. As in Fig. 3.3, the datum point in brackets was excluded from the regression analysis.

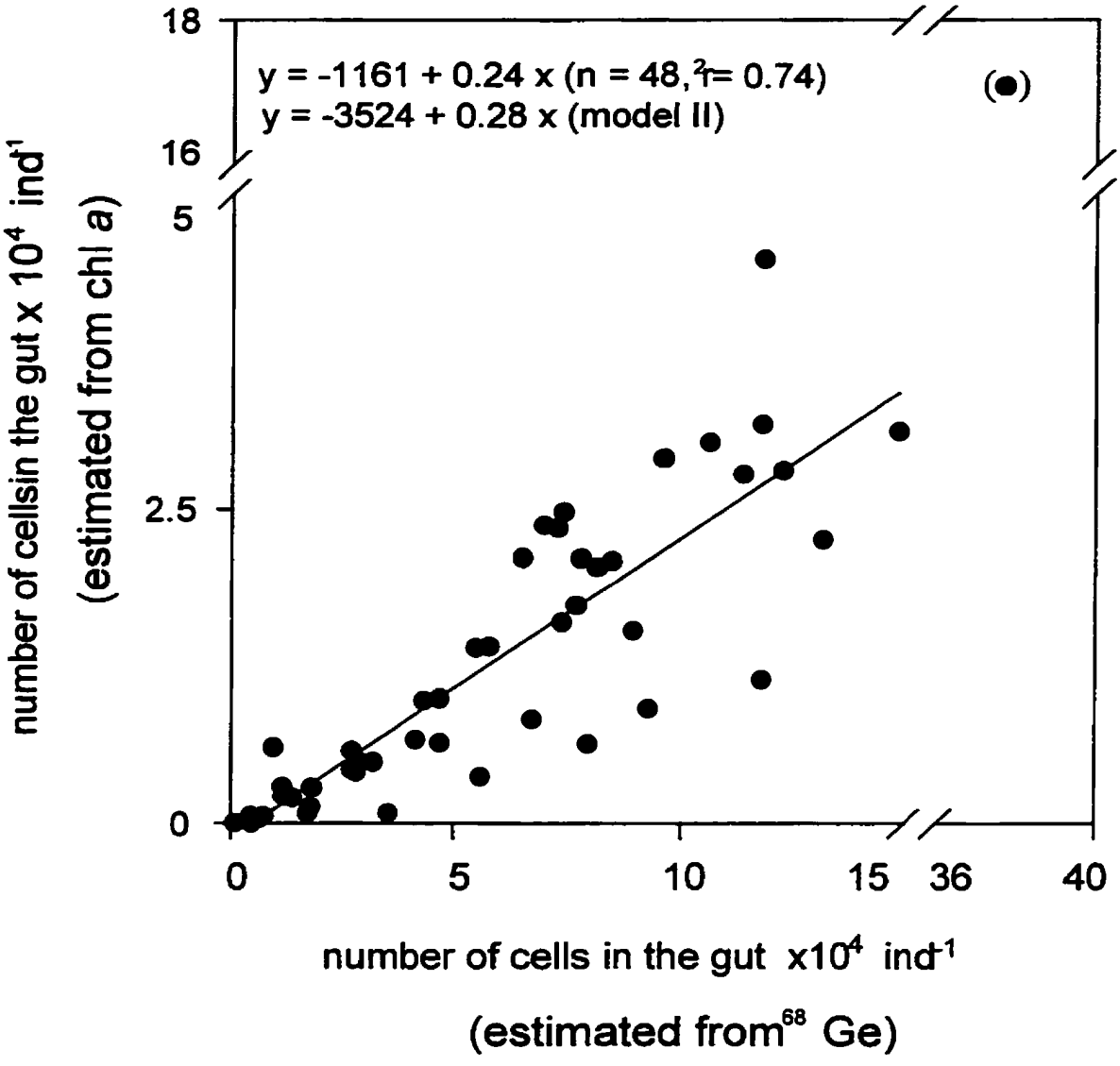

y = -1161 + 0.24 x (n = 48, r² = 0.74)
y = -3524 + 0.28 x (model II)
(●)
18
16
5
2.5
0
0
5
10
15
36
40
number of cellsin the gut x 10⁴ ind⁻¹
(estimated from chl a)
number of cells in the gut x10⁴ ind⁻¹
(estimated from ⁶⁸ Ge)

Fig. 3.5. The magnitude of chl *a* conversion in relation to the undegraded amount of chl *a* found in the animals. Data in brackets have been excluded from the regression analysis.

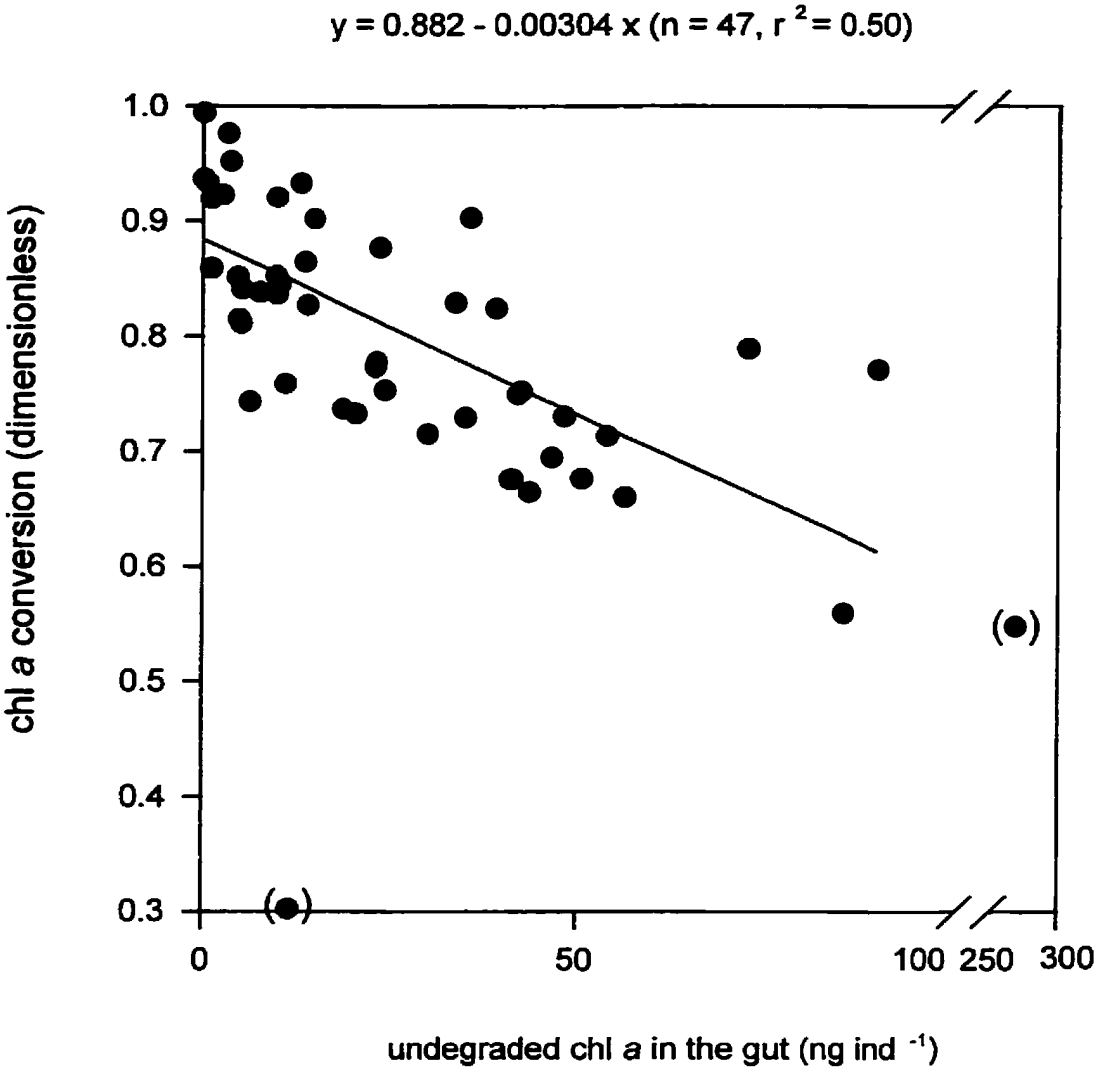
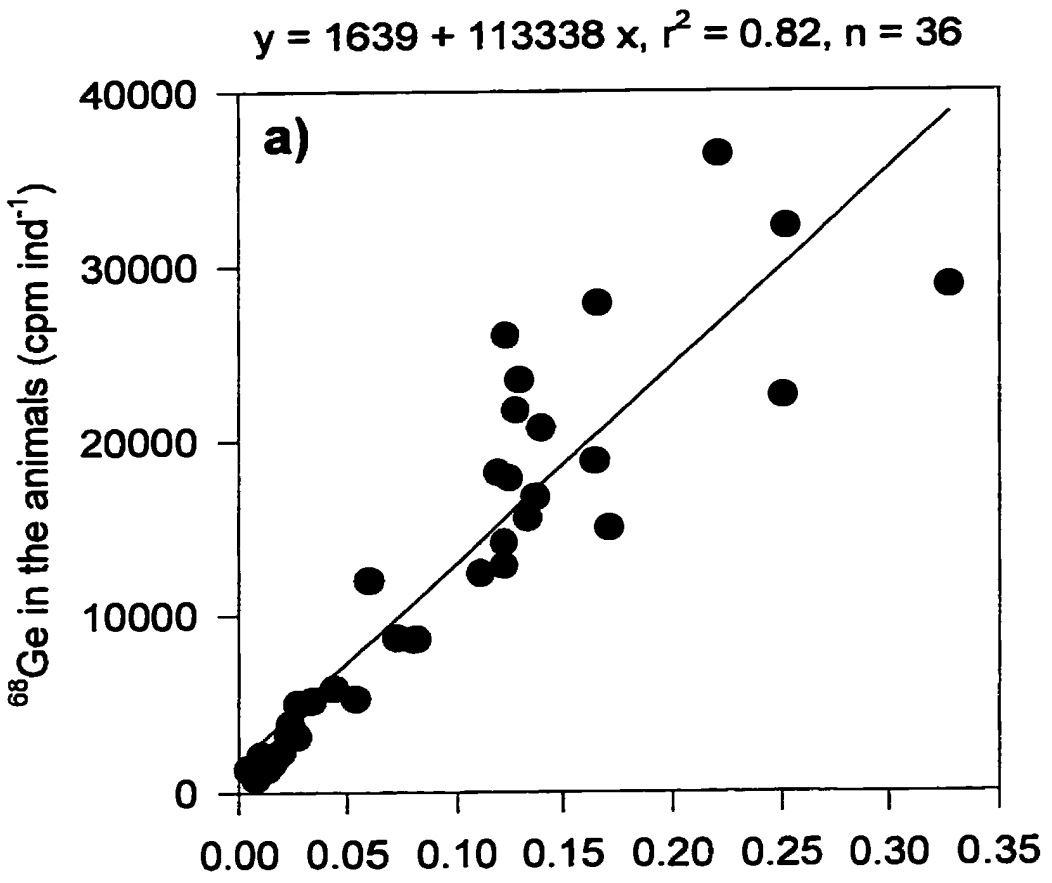

y = 0.882 - 0.00304 x (n = 47, r 2 = 0.50)
chl a conversion (dimensionless)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
50
100
250
300
undegraded chl a in the gut (ng ind -1)

Fig. 3.6. Relationship between $^{68}$Ge (a) and chl *a* (b) and the volume of food in the gut in the laboratory experiments as determined by video analysis. The volume was calculated from the linear dimensions of the food pellets in the gut and calculating prolate spheroid volumes.


y = 1639 + 113338 x, r² = 0.82, n = 36
a)
68Ge in the animals (cpm ind-1)
40000
30000
20000
10000
0
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35

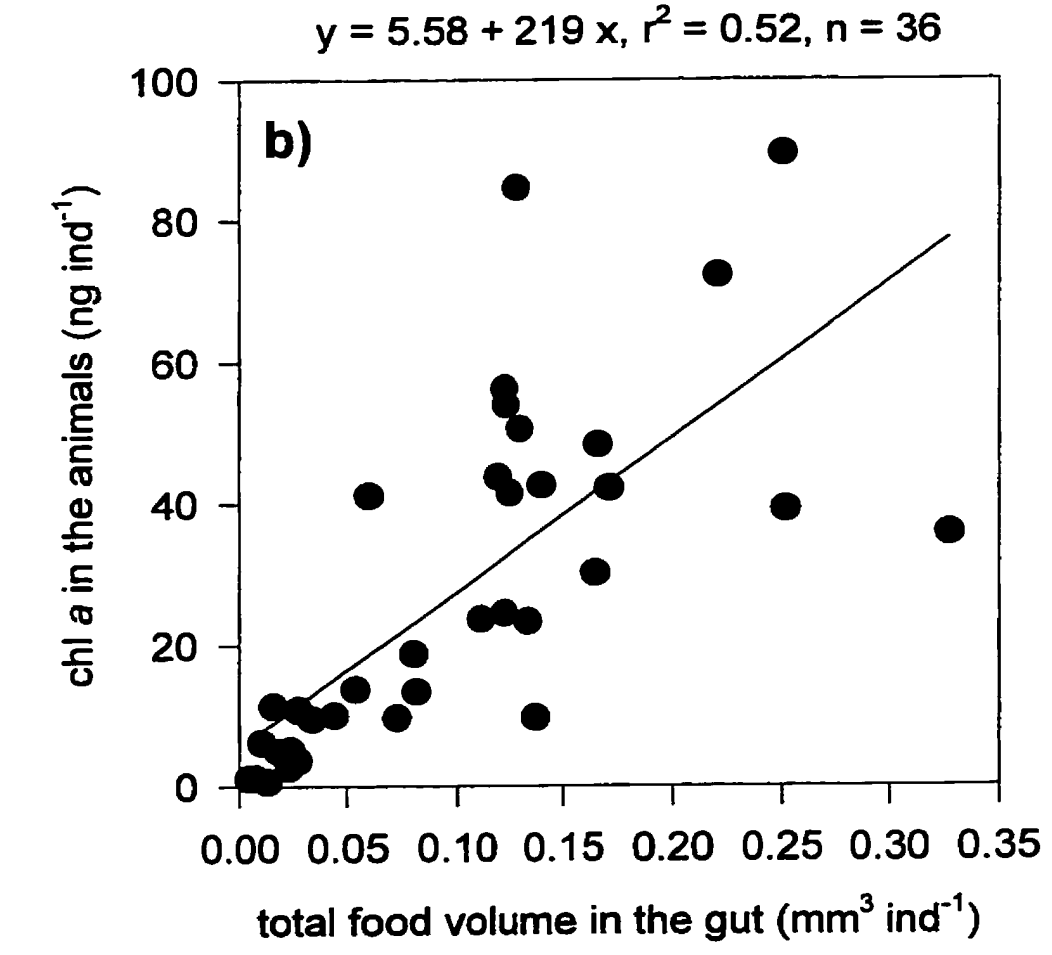
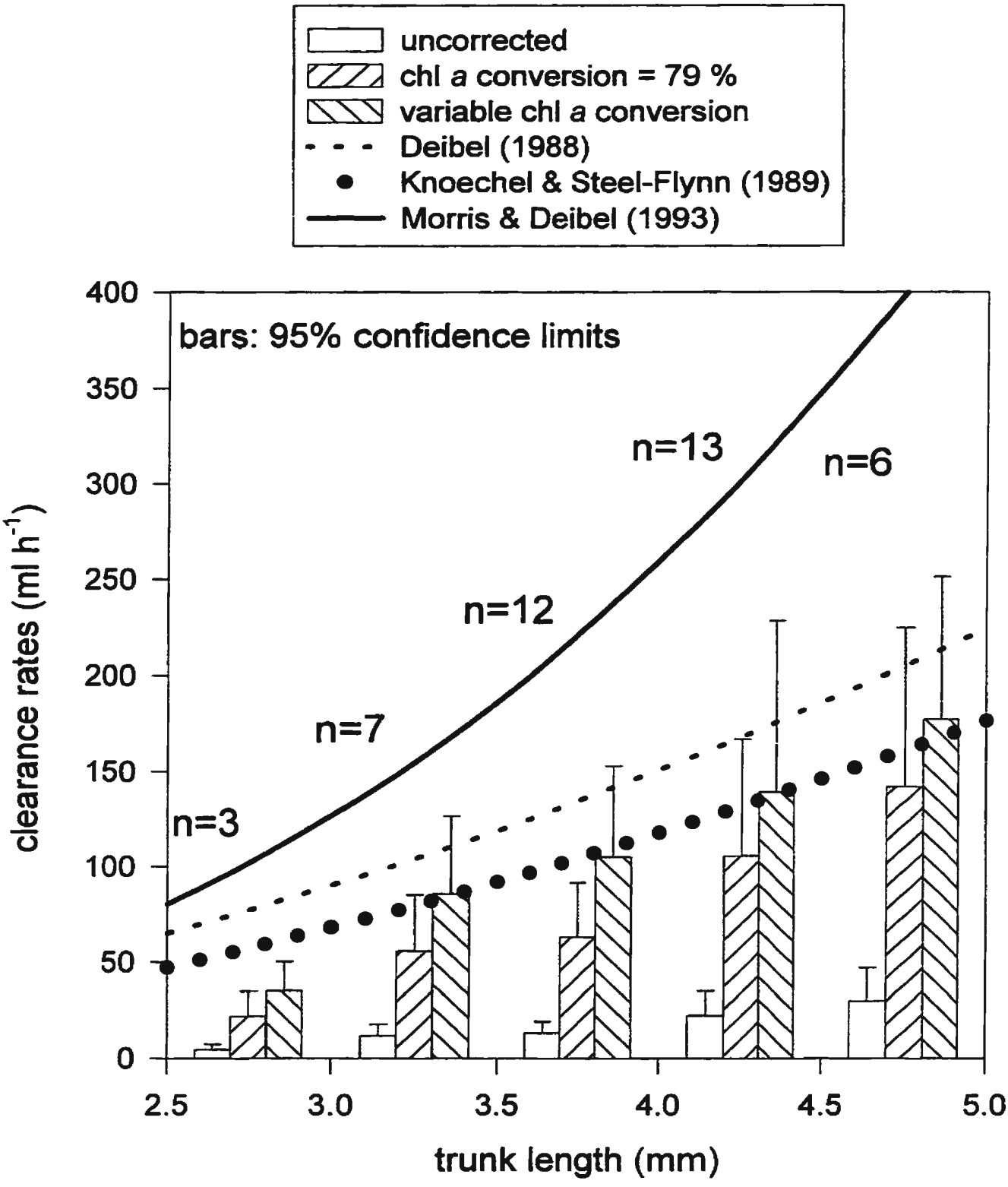

y = 5.58 + 219 x, r² = 0.52, n = 36
b)
chl a in the animals (ng ind-1)
100
80
60
40
20
0
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35
total food volume in the gut (mm3 ind-1)

Fig. 3.7. Clearance rates of *Oikopleura vanhoeffeni* as determined by the gut pigment technique and three published allometric equations. Deibel (1988) used the uptake of latex beads by the houses and animals to calculate clearance rates, and Knoechel and Steel-Flynn (1989) designed an *in situ* feeding device to measure the uptake of $^{14}C$ labelled diatoms. The highest clearance rates were derived from the ideal model of appendicularian filter feeding, assuming that the animals spent 50% of their time feeding (Morris and Deibel 1993). Clearance rates based on the gut pigment technique were calculated using a constant chl *a* conversion of 4.76 or by using 'variable conversion factors' based on the regression equation in Fig. 3.5. The open bars indicate clearance rates calculated on the basis of uncorrected chl *a* in the animals.


uncorrected
chl a conversion = 79 %
variable chl a conversion
Deibel (1988)
Knoechel & Steel-Flynn (1989)
Morris & Deibel (1993)
bars: 95% confidence limits
n=3
n=7
n=12
n=13
n=6
clearance rates (ml h-1)
trunk length (mm)
0
50
100
150
200
250
300
350
400
2.5
3.0
3.5
4.0
4.5
5.0

# Chapter 4. Assimilation efficiencies and biochemical fractionation of assimilated compounds in *Oikopleura vanhoeffeni*

## 4.1 Introduction

Quantifying organic matter assimilation is important in understanding feeding ecology. On an autecological level, the extent to which various organic nutrients are absorbed is important for growth and survival of individuals and may elucidate feeding and digestive strategies (Checkley 1980, Houde and Roman 1987, Kiørboe 1989, Roman 1991). On a synecological level, changes in the composition of particulate organic matter due to ingestion and digestion by major grazers are important for modifying biogeochemical fluxes, since pellets of large zooplankton are a primary mode of particle flux (e.g. Suess 1980, Michaels and Silver 1988, Fortier et al. 1994). The extent to which primary production is utilized and its organic nutrients assimilated by zooplankton determines how much of this material is recycled in the water column and how much of it is a food source for the benthos (Pilskaln and Honjo 1987, Falkowski et al. 1988, Aksnes and Wassmann 1993).

Pelagic tunicates are among the major grazers that remove a significant fraction of primary production from the water column (Alldredge 1981, Deibel 1988, Knoechel and Steel-Flynn 1989), produce fast-sinking fecal pellets (Michaels and Silver 1988, Fortier et al. 1994) and, in the case of appendicularians, discard a large number of fast-sinking

filtering devices or 'houses' (Taguchi 1982). While assimilation efficiencies have been studied in great detail for crustacean zooplankton, especially copepods (e.g. Conover 1966 a, b) and krill (e.g. Lasker 1960), information pertaining to pelagic tunicates is sparse. Gorsky (1980) examined assimilation efficiencies in the appendicularian *Oikopleura dioica*, Andersen (1986) in the salp *Salpa fusiformis* and Madin and Purcell (1992) in the salp *Cyclosalpa bakeri*. Assimilation efficiencies have not yet been determined for doliolids and pyrosomids (Madin and Deibel, in press.).

Several authors have incorporated biochemical information into assimilation studies of copepods (Roman 1991, Head 1992, Anderson 1994, Cowie and Hedges 1996). According to Anderson (1994), information on the biochemical composition of food and feces aids in the interpretation of the C:N ratios of particles in the water column and those mediating export. The protein fraction is of particular interest, since there is evidence that growth and fecundity of zooplankton are protein (i.e. nitrogen) limited (Checkley 1980, Kiørboe 1989). The residual biochemical composition is also important for benthic deposit feeders, since fecal pellets have a different nutritional quality than sinking phytoplankton (Cole et al. 1987). Finally, the degree to which organic compounds are utilized by animals may help to find suitable biomolecules as markers for organic matter (Cowie and Hedges 1996).

In the present study, the assimilation efficiency for bulk carbon by the cold water appendicularian *Oikopleura vanhoeffeni* feeding on the laboratory grown diatom *Thalassiosira nordenskioldii* was investigated. The term 'assimilation' is used loosely in the sense of absorption. Strictly, however, assimilation is absorption minus respiration which is equivalent to the absorption of carbon available for growth. I employed a dual-

labeling technique using the radio isotopes $^{14}C$ and $^{68}Ge$, a technique very similar to Tande and Slagstad's (1985) $^{14}C$:biogenic silica method. However, instead of determining the amount of biogenic silica chemically, $^{68}Ge$ was used to label the silica frustules. The method was rigorously tested and has many benefits over alternative techniques such as $^{14}C$ and biogenic silica (Tande and Slagstad 1985) or other dual-labeling techniques (Calow and Fletcher 1972). In addition to the determination of assimilation efficiencies of bulk carbon, the incorporated $^{14}C$ was fractionated into four major groups of biochemical constituents (i.e. low molecular weight compounds, lipids, proteins and polysaccharides) which allowed me to determine the digestibility of these four biochemical pools.

## 4.2 Materials and Methods

### Preparation of the experimental food source

The cold water diatom *Thalassiosira nordenskioldii* (equivalent spherical diameter = 15μm) was grown under continuous light at 5 °C in f/2+ medium. When a new medium was inoculated with algae, $^{68}Ge(OH)_4$ (New England Nuclear Corp.) and $NaH^{14}CO_3$ were added and the flask stoppered tightly to avoid any exchange with the atmosphere. The final concentrations were 100 μCi $l^{-1}$ for $^{14}C$ (Nielsen and Olsen 1989) and 60 μCi $l^{-1}$ for $^{68}Ge$ (Penry and Frost 1991). At these levels, the isotopes have no detectable effects on growth rates (Nielsen and Olsen 1989, Penry and Frost 1991). The algae were harvested in late exponential phase, 10 to 17 d after transfer. The long incubation times with the radio-isotopes ensured that the algae were uniformly labeled, for which at least 5 cell

doublings were recommended (Nielsen and Olsen 1989). Before the diatoms were added to the experimental jars, dissolved label in the culture was removed by a series of reverse flow filtrations using a 7μm screen, thereby diluting the label ca. 10,000-fold.

Assimilation experiments with *O. vanhoeffeni*

Animals were collected in 500 ml glass jars (one animal per jar) by shore-based SCUBA divers in front of the Ocean Sciences Centre (Logy Bay, Newfoundland) from January to June 1996 . The jars were immediately transported into the laboratory and placed in running sea water at ambient temperature. Within 3 h of collection, jars containing filtering animals were put on ice, old houses were removed with a wide-mouthed pipette, and the labeled algae added in various concentrations ranging from ca. 30 to 1330 cells $ml^{-1}$ final concentration in order to test the effect of availability of food on assimilation efficiency. The jars were covered with ice and kept in the dark for the entire experiment (2 - 4 h). At the end of the incubation, 200 ml of sea water were removed from the jars using a syringe, carefully avoiding fecal pellet contamination, and the water passed through a GF/F glass fiber filter. The animals were gently sucked into a wide-mouthed pipette through the escape chamber of the house (Flood 1991) and put into GF/F filtered sea water on ice. They were then serially transferred through two washes in GF/F filtered sea water to reduce the number of attached algae. The trunk length (excluding the gonads) of each animal was measured to the nearest 20 μm. Forceps were used to remove the animals by the tail from the filtered sea water and to transfer them into 7 ml glass scintillation vials. Fecal pellets were removed from the bottom of the jar and from the dissected houses with Pasteur pipettes, transferred twice into 10 ml of GF/F filtered sea water to remove attached algae, and pipetted onto a GF/F filter using as little

water as possible. Residual $NaH^{14}CO_3$ was removed by adding 0.25 ml of 0.2 N perchloric acid to the samples and leaving the vials loosely capped for 12 h. Five ml of scintillation cocktail (Ecolume) was then added and the vial stored for at least 48 hours before counting to ensure that $^{68}Ge$ attained a transient equilibrium with $^{68}Ga$ (Rivkin 1986). The samples were counted with a Packard Tri Carb liquid scintillation spectrometer (model 2500 TR) and corrected for background activity. Assimilation efficiencies were calculated using the formula (Tande and Slagstad 1985):

$$(1) \qquad AE_T = \left(1 - \frac{^{68}Ge_d/^{14}C_d}{^{68}Ge_f/^{14}C_f}\right) * 100$$

where $AE_T$ is the assimilation of total or bulk carbon, $^{68}Ge_d$ and $^{68}Ge_f$ are the dpm of $^{68}Ge$ in the diatoms and feces, respectively, and $^{14}C_d$ and $^{14}C_f$ the dpm of $^{14}C$ in diatoms and feces, respectively, at the end of the experiment.

Test of $^{68}Ge$ as a conservative tracer

For this experiment, three groups of animals were compared: one group was allowed to feed on the dual-labeled algae (ca. 50 cells $ml^{-1}$) and then placed in filtered sea water to evacuate their guts before measurement in the scintillation counter. A second group with full guts was transferred into scintillation vials, whereas a third group of animals with crippled tails were kept in the experimental jars in order to provide non-feeding controls. If $^{68}Ge$ is indeed a conservative tracer, the amount of tracer recovered

from the first group should be equal to that from the third group. For comparison, the second group shows how much $^{68}$Ge is present in animals with full guts.

Comparison with copepods

Applying a new method poses the risk that the values obtained are not comparable with other studies. Since no information exists on the assimilation efficiencies of cold water appendicularians, copepods, which have been studied in more detail, were measured in one experiment. For this purpose, a mixture of calanoid copepods (mainly *Calanus finmarchicus* and *C. glacialis* stage VI females) were collected with a plankton net. After two days in buckets filled with ambient sea water, the copepods were divided into two groups. One group was fed an unlabelled suspension of *T. nordenskioldii*, whereas the other group was kept in filtered sea water overnight. The following day, the animals were transferred into suspensions of dual-labeled algae and kept on a Ferris wheel overnight (12 h) at 2.5 °C. After settling for ca. 30 min, fecal pellets were removed from the bottom of the jar, transferred into well plates and washed three times with 10 ml of GF/F filtered sea water. At least 20 pellets were transferred onto each GF/F filter and each filter put into a glass scintillation vial. After adding 0.25 ml of 0.2 N perchloric acid and leaving the vials loosely capped for 12 h, scintillation cocktail was added. One hundred and eighty ml of the supernatant food suspension was removed with a syringe (avoiding fecal pellet contamination), filtered onto GF/F filters and processed in the same manner as the water samples from the *O. vanhoeffeni* experiments.

Dual-labeling protocol

The maximum β-energies are 156 keV and 1.9 MeV for $^{14}C$ and $^{68}Ge$, respectively. The ratio of these maximum energies (i.e. 12.2) is therefore high enough for complete separation of the two isotopes (Kobayashi and Maudsley 1974). For each isotope, standard quench curves were constructed using chloroform as the quenching agent and internal standards. For the dual labeling counting protocol, I followed the procedure outlined in the Packard Tri-Carb liquid scintillation analyzer operation manual. To test for accuracy of the technique, I prepared mixtures of known concentrations of $^{14}C$ and $^{68}Ge$ and measured them with the new dual-label scintillation counting protocol.

Biochemical fractionation

In one experiment, diatoms which were labeled solely with $^{14}C$ were fed to the animals. The remainder of the protocol was identical to the one for the dual labeling experiments (see above). Algae, animals and feces were fractionated into biochemical pools using the separation technique of Li et al. (1980) as modified by Rivkin (1985). Briefly, the four fractions were low molecular weight (LMW, water soluble), lipid (chloroform-methanol soluble), polysaccharides (hot TCA soluble) and proteins (TCA insoluble). The samples were dried at 50 - 60 °C, resuspended in 0.2 ml of distilled water and counted in 5 ml of Ecolume scintillation cocktail. GF/F filters without biological material were processed in the same manner and served as blanks. Recovery of $^{14}C$ after extraction (sum of $^{14}C$ in all biochemical pools in proportion to unextracted samples) is usually > 90% (Rivkin 1985).

## 4.3 Results

### Testing the dual-labeling protocol

A wide range of ratios of known amounts of $^{14}C$ and $^{68}Ge$ was used to test whether the isotopes can be accurately separated. The results (Fig. 4.1) were divided into two ranges, a lower range in which experiments were performed (Fig 4.1a), and an upper range in which the measured ratios depart considerably from linearity at higher $^{68}Ge$ concentrations (Fig. 4.1b). I tested the sensitivity of the assimilation rates to slight departures from the ideal 1:1 relationship shown in Fig. 4.1a. The calculation error for assimilation efficiencies was 2.5% in extreme cases and, considering other experimental sources of error, was thus negligible.

### Assimilation efficiencies for bulk carbon

Table 4.1 summarizes the five experiments with *O. vanhoeffeni* and the one experiment with copepods. The assimilation efficiencies were calculated for each animal and were significantly different among experiments with the means for each experiment ranging from 42 to 83 % (one-way ANOVA, Table 4.1). The differences in assimilation efficiencies among experiments (Table 4.1) were due to a significantly higher ratio of $^{68}Ge$:$^{14}C$ in the algae in experiment 4 ($n = 67$, $r^2 = 0.97$, $p < 0.0001$). In contrast, the ratio of $^{68}Ge$:$^{14}C$ in the fecal pellets was constant and not significantly different among experiments ($n = 49$, $r^2 = 0.05$, $p = 0.65$). The assimilation efficiencies were not related to

trunk length (Fig. 4.2a) nor gut content of the animals (Fig. 4.2b). The mean assimilation efficiency for the copepods was 74 % (± 8% SD) (Table 4.1). There was no significant difference between the means of prefed and starved copepods (one-way ANOVA, n = 8, 2 groups, p = 0.33).

Biochemical fractionation

The proportions of radiolabel in each biochemical fraction for each compartment (diatom, animal and feces) are shown in Fig.4.3. There was a significant difference in the proportions of all biochemical constituents between algae and fecal pellets (Wilcoxon 2-sample test). Non-parametric statistics were used since the residuals after pairwise comparison with a one-way ANOVA were not normally distributed, even after an arcsine transformation of the proportions (Shapiro-Wilk statistical test for normality, SAS statistical software).

## 4.4 Discussion

Test of assumptions

The following is a combination of conditions suggested by Calow and Fletcher (1972), Wightman (1975) and Tande and Slagstad (1985) which must be satisfied in order for a dual labeling method to give reliable results:

*Are $^{14}C$ and $^{68}Ge$ uniformly distributed throughout the food material?* Since only uniformly-labeled cell cultures were used in this study, this assumption was met. The algae were uniformly labeled because of the extremely long incubation times (>10 d, Nielsen and Olsen 1989).

*Does the digestible fraction of carbon derive entirely from food?* Since $^{14}C$, not ash free dry weight, was used to represent organic carbon, in contrast to the Conover ratio technique, all the labeled carbon which was recovered in the feces derived from the diatoms and not from mucous and chitinous intestinal secretions (Johannes and Satomi 1967).

*Does the $^{68}Ge$ : $^{14}C$ ratio change in the food during the feeding experiment?* To answer this question, subsamples of the cultures were taken before the experiments and compared with the water samples taken after the experiments (Table 4.1). There was essentially no change in the isotope ratios of *T. nordenskioldii* before and after the experiments, mainly due to the short incubation times (< 4h). For comparison, Tande and Slagstad (1985) found that *Thalassiosira anguste-lineata* lost only 10% of the initial $^{14}C$ activity within the first 24 h in the dark and at 0.5 °C.

*Are $^{14}C$ and $^{68}Ge$ moving through the gut at the same rates?* It has been shown by Bricelj et al. (1984) that the bivalve *Mercenaria mercenaria* has separate pathways for processing cell walls and cell contents of *Pseudoisochrysis paradoxa*. The cell contents are incorporated into the digestive gland and remain there for intracellular digestion. Thus $^{14}C$ is retained for a longer period of time than cell wall constituents, and because of this delay complete recovery of the feces may be important. For copepods, this does not

present a problem since intracellular digestion does not take place in crustaceans, although it does occur in some chordates such as *Amphioxus* sp. (Barnard and Prosser 1973). Although I do not know whether intracellular digestion occurs in pelagic tunicates, this potential problem was reduced by collecting almost all fecal pellets. The fecal pellets were therefore representative subsamples for the entire incubation period. Phagocytosis of whole cells, as reported by Hildreth (1980) for mussels, is unlikely because of the low amounts of $^{68}$Ge which I found within the animals (Table 4.2).

*Is $^{68}$Ge absorbed to any significant degree?* The dual labeling technique permits direct testing of one of the most fundamental assumptions of any technique which uses conservative tracers. Since individual animals can be measured, no indirect budget calculations based on assumptions of complete recovery, as performed by Tande and Slagstad (1985), were necessary to explore the potential absorption of $^{68}$Ge. The amount of $^{68}$Ge was ca. twice as high in animals after defecation than in the crippled controls (Table 4.2), but how do these values compare with the total amount of $^{68}$Ge which passed through the gut? Using the amount of $^{14}$C absorbed by the animals (14113 dpm, Table 4.2), one has a minimum estimate of the amount of algae which must have passed through the gut during the incubation period. This is considered a minimum estimate because it does not account for respiration or incomplete digestion. Using an average ratio of $^{68}$Ge and $^{14}$C in the algae (i.e. 0.07, Table 4.1), the amount of $^{68}$Ge which passed through the guts during the incubation period was 988 dpm. The amount retrieved in the animals after gut evacuation (39.48 dpm minus blank) was only 21 dpm, or 2% of 988 dpm (Table 4.2). This shows that even under the extreme case that 100% of carbon was absorbed, and that no respiration occurred, a negligible amount of $^{68}$Ge was taken up by the animals. I therefore conclude that $^{68}$Ge incorporated into the silica frustules of diatoms is a useful

conservative tracer and that the 15% loss of biogenic silica as calculated by Tande and Slagstad (1985) is probably due to incomplete recovery of feces in their experiments.

*Does $^{68}Ge$ or $^{14}C$ leak from the fecal pellets?* Since $^{68}Ge$ is incorporated into the silica frustules that are wrapped within a fecal pellet membrane, it is unlikely that $^{68}Ge$ leaks from the pellets. Furthermore, unlike coprophagous copepods, *O. vanhoeffeni* can neither feed on nor mechanically rupture fecal pellets. On the other hand, dissolved $^{14}C$ can potentially pass the fecal pellet membrane. According to Jumars et al. (1989), one would expect that more than 50% of any dissolved substance would diffuse out of a fecal pellet within 5 min. However, one can also assume that substances which quickly diffuse would also be efficiently absorbed by the gut walls of the animals before egestion.

*Does coprophagy occur?* If, for a given animal, the probability of ingestion of its own feces is higher in an experimental condition than it is in nature, the assimilation efficiencies could be artificially elevated. This is particularly a problem for copepods kept at high densities in incubation jars (compare Lampitt et al. 1990). Deposit feeders are known to ingest their own feces to maximize extraction of organic nutrients from the food (Mason and Odum 1969). *O. vanhoeffeni,* however, cannot reingest its fecal pellets since the inlet filter meshes exclude particles of this size (Deibel 1986).

*Is the experimental situation representative of the field?* Assimilation efficiencies are affected by the quality of the food (Houde and Roman 1987) and by acclimation of animals to the quantity of food (Landry et al. 1984). Maintaining animals in experimental conditions inevitably introduces the risk of producing results which are not representative of the field. However, for assimilation studies in general, the potential for laboratory

artifacts is usually outweighed by the advantage of more controlled conditions of *in vitro* studies. To minimize experimental artifacts, I processed *O. vanhoeffeni* within hours of collection, thereby ensuring that the animals were healthy and fed. Diatoms in general constitute a major part of the diet of *O. vanhoeffeni* since large amounts of chlorophyll *c* are found in their guts throughout the field season (chapter 3).

Comparison of the $^{68}Ge$:$^{14}C$ dual labeling technique with other methods

The Conover ratio technique (1966a) has been applied in most assimilation studies on zooplankton. Since differences in weight of food and feces before and after combustion are used to determine the organic content of the samples, relatively large amounts of material are required to achieve accurate results (Navarro and Thompson 1994). This method is therefore restricted to animals which are either large enough to produce large fecal pellets, or to animals which can be collected in large numbers. *O. vanhoeffeni*, however, must be collected individually by SCUBA diving. Since only a small number of individuals can be processed in each experiment, the amount of fecal material is small. Radiolabeling diatoms with $^{68}Ge$ makes the method very sensitive in comparison. Although 2 to 13 pellets were processed per assay, single fecal pellets would be sufficient, since the signal from $^{68}Ge$ incorporated into as little as a single diatom cell can be detected (Rivkin 1986). Another disadvantage of the Conover ratio is that it is sensitive to products added to the feces which are derived from the animals. Copepods, for example, wrap their feces in chitinous membranes (Nott et al. 1985), whereas fecal pellets of pelagic tunicates contain undigested pharyngeal filters (Deibel and Turner 1985). Depending on whether the added material is a component of the ash or the organic

fraction, assimilation efficiencies would then be over- or underestimated, respectively. Finally, another criticism of the Conover ratio is that even part of the 'ash' is not as indigestible as originally assumed. It has been found for prawns that more than 30% of the inorganic fraction is absorbed, which could lead to underestimation of assimilation efficiencies (Forster and Gabbott 1971). Both the secretion of material and the digestion of the ash component of the food may not be significant, but these factors warrant further examination. The dual labeling technique in this study is free from these problems.

A dual labeling (or twin tracer) technique for assimilation experiments has already been described by Calow and Fletcher (1972) using $^{51}Cr$ as the indigestible fraction. The advantage of $^{51}Cr$ is that it is not restricted to diatoms and can be used for a variety of food sources. However, considerable amounts of tracer can be lost since $^{51}Cr$ is not incorporated into the cells like $^{68}Ge$, but absorbed to the surface (Stuart et al. 1982). This is particularly problematic in filter feeders like bivalves, in which losses onto the large surface areas of ctenidia, palps and digestive glands have been reported to be as high as 17 to 45 % (Stuart et al. 1982) and 14 % (Bricelj et al. 1984) of the ingested amount.

Biogenic silica has been used as a conservative tracer for assimilation studies (e.g. (Tande and Slagstad 1985, Head 1992, Cowie and Hedges 1996) as well as to investigate pigment destruction (Conover et al. 1986, Head 1988). However, measuring biogenic silica instead of the incorporated $^{68}Ge$ has several disadvantages. First, samples must be split into two parts, one of which is used for analysis of the organic or pigment fraction, and the other is hydrolyzed prior to performing the silica assay. Therefore, more material is required and more analytical steps are involved. In the dual labeling approach with $^{68}Ge$, the feces, as well as the filtered algae, are simply transferred into scintillation vials.

Fewer analytical steps mean less chance for contamination or loss of material. One could argue that carbon to biogenic silica ratios could be studied in the field, as attempted by Head (1992) to estimate assimilation efficiencies, but the ratios of food and feces can only be compared if feeding is unselective. In copepods this is rarely the case, and one of the fundamental assumptions (see above) is therefore violated. For animals with some degree of selectivity, including various retention efficiencies of mucous filters, the biogenic silica assay and the $^{68}Ge$ : $^{14}C$ dual labeling approach are therefore limited to laboratory studies with diatoms.

Assimilation of bulk carbon

The assimilation efficiencies of *O. vanhoeffeni* varied significantly among experiments (Table 4.1). It is remarkable that this difference of assimilation efficiencies was not due to a change in isotope ratios in fecal pellets, but rather in the isotope ratios of the diatoms. This may be explained by a change in the organic composition of the cells. For example, diatoms can accumulate polysaccharides such as acid soluble β-1,3 glucan (i.e. chrysolaminaran, Darley 1977) during the later stages of their growth (Myklestad 1974). According to Reinfelder and Fisher (1991), the cell plasma rather than the cell wall components of diatoms are digested by copepods. If a similar mechanism holds true for *O. vanhoeffeni* and the high ratio of $^{68}Ge$ : $^{14}C$ in experiment 4 was a reflection of relatively small amounts of digestible carbon in the cell plasma, the low assimilation rates could be explained. As a consequence, it is mainly the cell wall constituents that would be defecated and, since they are less variable than the plasma carbon (Handa 1969), the $^{68}Ge$:$^{14}C$ in the feces would be predictably less variable as well (Table 4.1). However, to

test this hypothesis the contribution of cell wall constituents to the polysaccharide fraction needs to be analyzed in more detail.

Comparison with other zooplankton

Assimilation efficiencies have been calculated by Gorsky (1980) for *Oikopleura dioica* using the Conover ratio (1966a) and assuming that various food particles were distributed in the same proportion in the gut as in the water. Three different food types were offered: (1) *Isochrysis galbana*, (2) a mixture of the diatom *Thalassiosira pseudonana* and the flagellate *Platymonas sueica* and (3) natural sea water filtered through a 50 μm screen. The assimilation efficiencies varied from 17% for *I. galbana* to 88% for the mixture of diatom and flagellate and 79% for the natural sea water. In comparison, the average value of 67% for assimilation efficiency is considerably lower than that calculated for the mixture containing the diatom in the *O.dioica* experiments. For *Salpa fusiformis* fed with the diatom *Phaeodactylum tricornutum* and the flagellate *Hymenomonas elongata*, Andersen (1986) determined assimilation efficiencies of 32% and 64%, respectively. The data in Gorsky (1980) and Andersen (1986) show opposite trends. While the food diatom was assimilated most efficiently and the flagellate least efficiently in the appendicularian, the opposite was true for the salp. Madin and Purcell (1992) calculated assimilation efficiencies of 61% for carbon and 71% for nitrogen for *Cyclosalpa bakeri* feeding on mixed phytoplankton containing diatoms. Since chl *a* is efficiently digested in the tunicate gut (chapter 3), chl *a* concentration was corrected for pigment loss by counting fragments of silica frustules in the feces (Madin and Purcell 1992). Assuming that most nitrogen is found only in the protein fraction (see below), the

assimilation efficiency for nitrogen in this study was ca. 72% (Table 4.3), similar to the one reported by Madin and Purcell (1992). The assimilation efficiency obtained for the copepods (i.e. 74%, Table 4.1) not only fits well within the range reported in the literature (summary Table 7.4 in Omori and Ikeda 1984), but also compares well with the assimilation efficiencies usually assumed for herbivorous zooplankton in ecosystem models (e.g. 75% for nitrogen, Fasham et al. 1990; 70% for carbon Falkowski et al. 1988, Frost 1993).

No effect of levels of ingestion on assimilation efficiencies

Beklemishev (1962) hypothesized that zooplankton would use food inefficiently when offered at high concentrations, and indeed some digestion models predict that assimilation efficiencies decrease with increasing gut fullness (Slagstad and Tande 1981). However, in many cases little or no effect of ingestion rates on the assimilation rates has been shown experimentally (e.g. Conover 1966b, Tande and Slagstad 1985). The assimilation efficiencies in this study were not affected by the amount of material in the guts measured as the amount of *T. nordenskioldii* recovered in the animals (Fig. 4.2b). Although natural seston was still present in the jars, it was greatly reduced by the filtering activity of the animals in the few hours before *T. nordenskioldii* was added. The size and color of the fecal pellets containing *T. nordenskioldii* were markedly different (brown-green) from the smaller black fecal pellets produced before the addition of the algae. One can therefore conclude that the amount of available food was closely related to the amount of *T. nordenskioldii* added to the suspension at the beginning of the experiments. Since gut passage times remain constant irrespective of the amount of food ingested, the gut content can be used as a proxy for ingestion rates (chapters 1 and 3). Assimilation

efficiencies were therefore not related to ingestion rates either. However, the lowest and some of the highest assimilation efficiencies were found in the lower gut content range (Fig. 4.2b). Reduced accuracy of the method at low gut content, possibly due to some diatoms being attached to the body surface of the animals, may have significantly contributed to the increased variance around the mean assimilation efficiency. Conover (1966b) faced a similar problem at low ingestion rates. He excluded these data points 'as points of doubtful significance', but still did not demonstrate a significant relationship between assimilation efficiency and ingestion rate. When I excluded gut content levels less than 25 dpm above the blank, the low assimilation values were eliminated, although assimilation efficiency and gut content remained independent ($n = 35$, $r^2 = 0.03$, $p = 0.32$). The removal of these data points would have little effect on the overall assimilation efficiency. The assimilation efficiency calculated as the grand mean of the experimental means would only increase from 67 (shown in Table 4.1) to 70%. A constant assimilation efficiency at various ingestion rates has two important implications: (1) using one value only for assimilation efficiency simplifies ecosystem models, and (2) it shows that enzyme systems in the guts of zooplankton are not saturated with substrate, even at high ingestion rates (compare Bochdansky and Deibel 1997). For appendicularians, as well as for copepods, these high levels of enzyme activity therefore ensure that incoming substrate is efficiently utilized whenever available (Hassett and Landry 1983).

## Biochemical composition of *T. nordenskioldii*

The four fractions extracted represent a very crude biochemical characterization of the pools of cell carbon. The LMW fraction consists of a mixture of water soluble metabolites such as monosaccharides, organic acids, amino acids and lipid precursors

(Laws 1991, Roman 1991). The polysaccharide fraction, on the other hand, ranges from structural polysaccharides to nucleic acids, which are also TCA soluble (ca. 5% of cell carbon, Laws 1991). In diatoms, the 'protein fraction' may contain as much as 33% chitin, as demonstrated for *Thalassiosira pseudonana* (Smucker and Dawson 1986). In uniformly labeled cells, the proportion of total carbon that is labeled is the same in all biochemical fractions and the distribution of $^{14}C$ among fractions should reflect the proportions of biochemical fractions in the cell. In this study, *Thalassiosira nordenskioldii* allocated an average of 17% of $^{14}C$ in the LMW fraction, 18% in the lipid fraction, 54% in protein and 11% in polysaccharides (Fig. 4.3). Assuming that most of the LMW compounds are simple sugars (Laws 1991), one arrives at 54% protein, 28% carbohydrate and 18% lipid, which is similar to 60% protein, 34% carbohydrate and 7% lipid as given in Parsons (1961). However, lipid levels in diatoms may be as high as 30% (Table 4 in Laws 1991). Using the same extraction technique, Rivkin (1985) arrived at 15 - 30% LMW, 22 - 35% lipid, 33 -48% protein and 7 - 10% polysaccharides for three diatoms. In summary, the partitioning of carbon among the various pools in *T. nordenskioldii* is representative for a wide variety of diatoms.

Assimilation efficiencies of various biochemical fractions in *O. vanhoeffeni*

A knowledge of the biochemical composition of food and feces, in conjunction with the assimilation efficiency of bulk carbon, allows me to calculate assimilation efficiencies for each biochemical compound (Fig. 4.4):

(2) $$AE_x = 100 - (100-AE_T)\, pf_x\, pd_x^{-1},$$

where $AE_x$ is the assimilation efficiency of the biochemical fraction (x) in %, $pf_x$ and $pd_x$ are the proportions of fraction x in the feces and diatoms, respectively, and $AE_T$ is the assimilation efficiency of bulk carbon in %, as measured by the dual labeling protocol. The assimilation efficiencies for each fraction derived using this formula are given in Table 4.3.

The assimilation efficiencies were highest for LMW and proteins, which is similar to the findings for copepods (Landry et al. 1984, Hassett and Landry 1988, Roman 1991). Copepods may maximize protein uptake since it could be limiting to growth and egg production (Checkley 1980, Houde and Roman 1987), although higher assimilation efficiencies for proteins may simply reflect the digestibility of nitrogenous compounds (Anderson and Hessen 1995). For lipids, the assimilation efficiencies were lower than for mean carbon in *O. vanhoeffeni*. Since this species does not accumulate lipids as storage products (Deibel et al. 1992), it may incorporate some into somatic tissue, use them for maintaining membrane fluidity (Deibel et al. 1992), or catabolize them as do krill (Pond et al. 1995). The high amounts of $^{14}C$ in the LMW fraction of the animals cannot entirely be explained by the preferential uptake of LMW (Fig. 4.3, Table 4.3), but may be attributable to the action of exo-enzymes on polysaccharides and proteins before the metabolites were absorbed by the animals. Finally, polysaccharides were expected to be the least digestible fraction, since a major portion of undigestible cell wall compounds would be located in this fraction (McClintock 1986, Reinfelder and Fisher 1991).

C:N ratios of *T. nordenskioldii* and fecal pellets

One of the benefits of biochemically fractionating phytoplankton and feces in assimilation studies is to predict the C:N ratios of sinking material. According to Laws (1991), the N:C ratio (by weight) can be calculated by dividing the proportion of $^{14}C$ allocated to protein by 2.8. An alternative calculation is to use mass fractions of standard carbohydrate, lipid and proteins as given in Gnaiger and Bitterlich (1984). However, for this calculation it must be assumed that all nitrogen is in the protein fraction. This assumption is violated by the fact that amino acids can be found in the LMW fraction and nucleic acids in the polysaccharide fraction (see above), but this problem may be of little significance. Gnaiger and Bitterlich (1984) have calculated a new nitrogen mass fraction of 0.173 for standard proteins of bacteria, algae and aquatic animals, a value which is significantly higher than the traditionally used mass fraction of 0.16. A third formula is given by Anderson (1994), who suggested dividing the C:N ratio of proteins (i.e. 3.96 by atoms) by the proportion of proteins in the sample. The results of the three calculations are given in Table 4.4 for diatoms and feces.

The C:N ratios ranged from 5.2 to 6.3 by weight for *T. nordenskioldii*, which is similar to the Redfield ratio of 5.7 (6.6 by atoms, DiTullio and Laws 1986) and the predicted ratio for phytoplankton of 6.5 by weight (Laws 1991). However, the C:N ratios of the fecal pellets of *O. vanhoeffeni* ranged from 6.0 to 7.2 by weight (depending on the type of calculation), and were at the lower end of the range measured for zooplankton feces (i.e. 6.7 - 19, Anderson 1994). This suggests that although there was a preferential assimilation of proteins over other fractions in *O. vanhoeffeni* (Table 4.3), there would only be a small increase in C:N ratios during digestion (Table 4.4). When generalizing the

dynamics of C:N ratios due to the feeding activity of zooplankton, it is important to keep in mind that in about half of the studies cited by Anderson (1994, his Table 3) C:N ratios either did not change much or even decreased from food to feces.

### 4.5 Conclusions

*O. vanhoeffeni* assimilates bulk carbon of the diatom *T. nordenskioldii* with an efficiency of 67%, independent of body size and ingestion rate. This was similar to 74% as determined for a mixture of copepods (*C. finmarchicus* and *C. glacialis*) with the same technique. Although one must exercise some caution when comparing these values with other studies using different techniques, the assimilation efficiencies fit well within the ranges reported for both herbivorous copepods and pelagic tunicates. There is no *a priori* reason to assume that assimilation efficiencies are different in crustaceans and tunicates when the same material is digested, since physiological processes are very similar in terms of carbon-based budget analyses (Schneider 1992). A 67% assimilation of bulk carbon is also similar to the 79% chlorophyll conversion reported in chapter 3, which supports for appendicularians as well as bivalves the suggestion that chlorophyll may be representative of the digestible fraction of ingested organic material (Navarro and Thompson 1994). *O. vanhoeffeni* preferentially assimilates proteins and LMW compounds over lipids and carbohydrates. However, the estimated C:N ratios of fecal pellets are not much lower than those of the diatoms, which puts them into the lower range of C:N ratios of fecal material determined for other zooplankton.

Table 4.1. Assimilation experiments with *Oikopleura vanhoeffeni* and *Calanus* spp. using the $^{68}Ge:^{14}C$ dual labelling technique. Ambient temperature is the temperature at the collection site in Logy Bay (St. John's, Newfoundland). Experimental temperature for *O. vanhoeffeni* was kept constant by covering the experimental jars with ice. Age of culture is the time from inoculation to the time of harvest of the culture. '$^{68}Ge:^{14}C$ *T. n.* before' and '$^{68}Ge:^{14}C$ *T. n.* after' are the ratios of the two isotopes in *Thalassiosira nordenskioldii* before and after incubation with animals, respectively. Values are given as means (sample size; ± standard deviation). 'overall' gives the mean of the means of each experiment with *O. vanhoeffeni*.'*' indicates the date of collection during the spring diatom bloom. N. B. The final column (8) is not derived directly by columns (6) and (7) but rather by using $^{68}Ge:^{14}C$ ratios for feces of each individual animal.

*Oikopleura vanhoeffeni*:

| date | ambient temperature (°C) | experim. temperature (°C) | age of culture (d) | $^{68}Ge:^{14}C$ *T. n.* before [mean (n; ±SD)] | $^{68}Ge:^{14}C$ *T. n.* after [mean (n; ±SD)] | $^{68}Ge:^{14}C$ feces [mean (n; ±SD)] | AE% |
|---|---|---|---|---|---|---|---|
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
| 13 Feb '96 | -1.2 | 0 | 14 | 0.046 (3; 0.0028) | 0.048 (12; 0.0041) | 0.36 (10; 0.17) | 83 (10; 7.7) |
| 14 Feb '96 | -1.2 | 0 | 15 | 0.049 (3; 0.0004) | 0.047 (12; 0.0061) | 0.26 (7; 0.18) | 75 (7; 13) |
| 6 May '96* | n.d. | 0 | 10 | 0.069 (3; 0.0007) | 0.069(21; 0.0047) | 0.30 (16; 0.19) | 68 (16; 19) |
| 31 May '96 | +4.0 | 0 | 17 | 0.118 (3; 0.0016) | 0.121 (10; 0.0038) | 0.32 (7; 0.26) | 42 (7; 31) |
| 14 June '96 | +4.5 | 0 | 15 | 0.061 (5; 0.0001) | 0.063 (12; 0.0041) | 0.23 (9; 0.14) | 66 (9; 15) |
| overall | | | | 0.069 | 0.070 | 0.29 | 67 |
| *Calanus finmarchicus* and *C. glacialis*: | | | | | | | |
| 25 Apr '96 | +1.5 | +2.0 | 11 | 0.089 (3; 0.001) | 0.084 (4; 0.003) | 0.326 (8; 0.021) | 74 (8; 1.8) |

Table 4.2. Test of whether $^{68}Ge$ is a conservative tracer. 'crippled' indicates animals whose tails had been crippled so that they could not ingest food particles. 'gut empty' animals were animals which had been fed on a suspension of labelled diatoms but were then kept in filtered seawater for ca. 2 h to empty their guts. For comparison, 'gut full' indicates animals with full guts which had been feeding and which were put into the scintillation vials. There was a significantly higher $^{68}Ge$ activity in the animals with empty guts compared with the crippled controls (one-way ANOVA, $F = 5.95$, $p = 0.025$, $n = 20$). However, the amount of $^{68}Ge$ retrieved in the animals was small in comparison with the amount of $^{68}Ge$ which must have passed through the animals according to the $^{14}C$ absorption data.

| treatment | $^{14}C$ (dpm) (n; ±SD) | $^{68}Ge$ (dpm) (n; ±SD) | $^{14}C$ : $^{68}Ge$ ratio |
|---|---|---|---|
| crippled | 111 (8 ; 138) | 19 (8; 4.9) | 6 |
| gut empty | 14113 (12; 12556) | 39 (12; 23) | 357 |
| gut full | 26898 (19; 26154) | 746 (19; 688) | 36 |

Table 4.3. Assimilation efficiencies for each of the four biochemical fractions using an average assimilation efficiency for bulk carbon of 67% in equation 2.

| fraction | $AE_X$ (%) |
|---|---|
| LMW | 71 |
| Lipid | 58 |
| Protein | 72 |
| Polysaccharide | 55 |

Table 4.4. Estimated C:N ratios for *Thalassiosira nordenskioldii* and feces of *Oikopleura vanhoeffeni* feeding on this diatom, calculated from proximate biochemical composition using three different methods (Gnaiger and Bitterlich 1984, Laws 1991 and Anderson 1994). For comparison, C:N ratios are given both by weight and by atoms.

| | | C:N by weight (atoms) | | |
|---|---|---|---|---|
| | % protein | Gnaiger (1984) | Laws (1991) | Anderson (1994) |
| *T. nordenskioldii* | 54 | 5.9 (6.9) | 5.2 (6.1) | 6.3 (7.3) |
| feces of *O. vanhoeffeni* | 47 | 6.9 (8.1) | 6.0 (7.0) | 7.2 (8.4) |

## 4.7 Figures

Fig. 4.1. Test of whether $^{68}Ge$ and $^{14}C$ can accurately be separated by the dual labelling protocol. The lower range (a) is best described by a linear regression and departs slightly from the ideal 1:1 line. However, at higher ratios of $^{68}Ge$ : $^{14}C$, the measured ratios depart significantly but predictably from the true ratios and would then have to be corrected by the formula shown in (b). All the experimental ratios were within the linear portion of the curve and no correction was required (shaded bar). Note that a) is plotted on a log scale and b) on a linear scale.

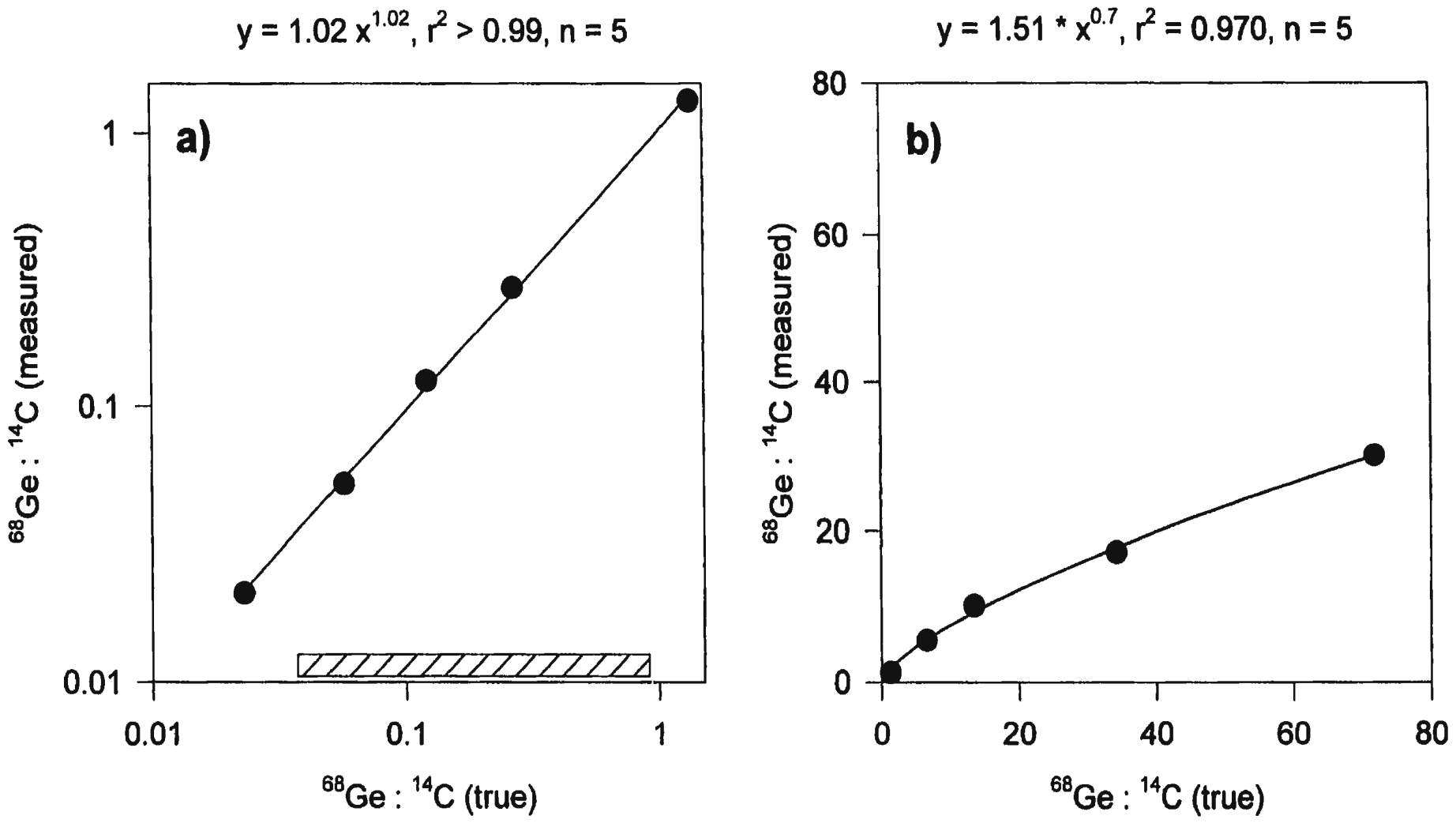

y = 1.02 x^1.02, r^2 > 0.99, n = 5
a)
1
0.1
0.01
0.01
0.1
1
68Ge : 14C (measured)
68Ge : 14C (true)
y = 1.51 * x^0.7, r^2 = 0.970, n = 5
b)
80
60
40
20
0
0
20
40
60
80
68Ge : 14C (measured)
68Ge : 14C (true)

Fig. 4.2. Assimilation efficiencies in *Oikopleura vanhoeffeni* were independent of (a) trunk length ($n = 43$, $r^2 = 0.081$, $p = 0.07$) and (b) amount of food in the gut ($n = 42$, $r^2 = 0.013$, $p = 0.47$). At constant gut passage times, gut fullness can be used as a proxy for ingestion rates (chapter 1 and 3).

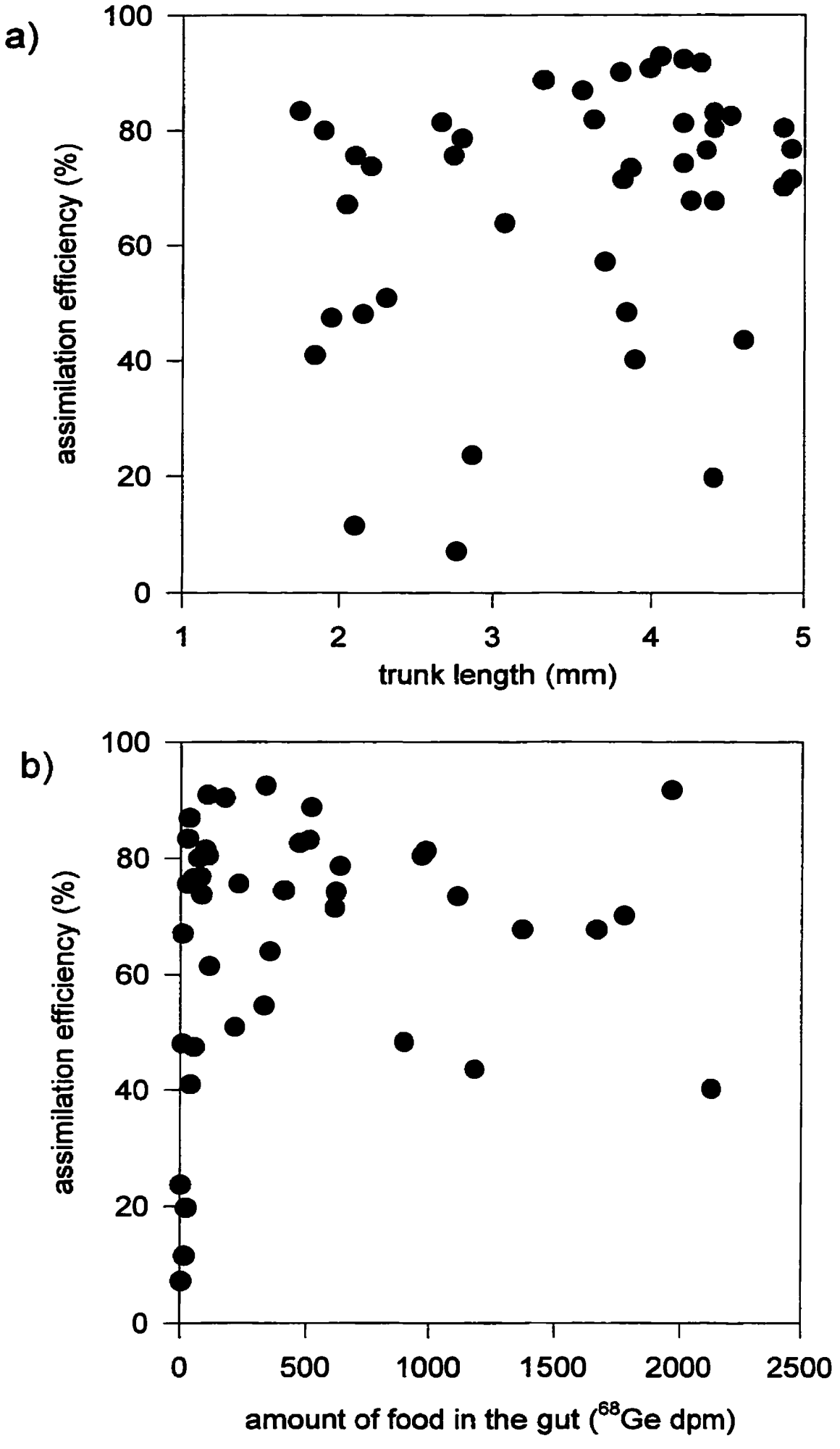

a)
assimilation efficiency (%)
100
80
60
40
20
0
1
2
3
4
5
trunk length (mm)
b)
assimilation efficiency (%)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
amount of food in the gut ($^{68}$Ge dpm)

Fig. 4.3. Proportions of each biochemical fraction for diatoms, animals and feces. Bars indicate standard deviations. In pairwise comparisons, the proportions changed significantly between diatoms and feces according to a Wilcoxon two-sample test. For LMW and proteins the proportions significantly decreased from food to feces. For lipids and polysaccharides the proportions increased from food to feces. a) $p = 0.034$, b) $p = 0.0012$, c) $p = 0.0063$, d) $p = 0.005$. The sample size was 33 in all fractions. Note the accumulation of LMW in the animals (a) in contrast to the low amounts of $^{14}C$ in proteins (c) and polysaccharides (d).

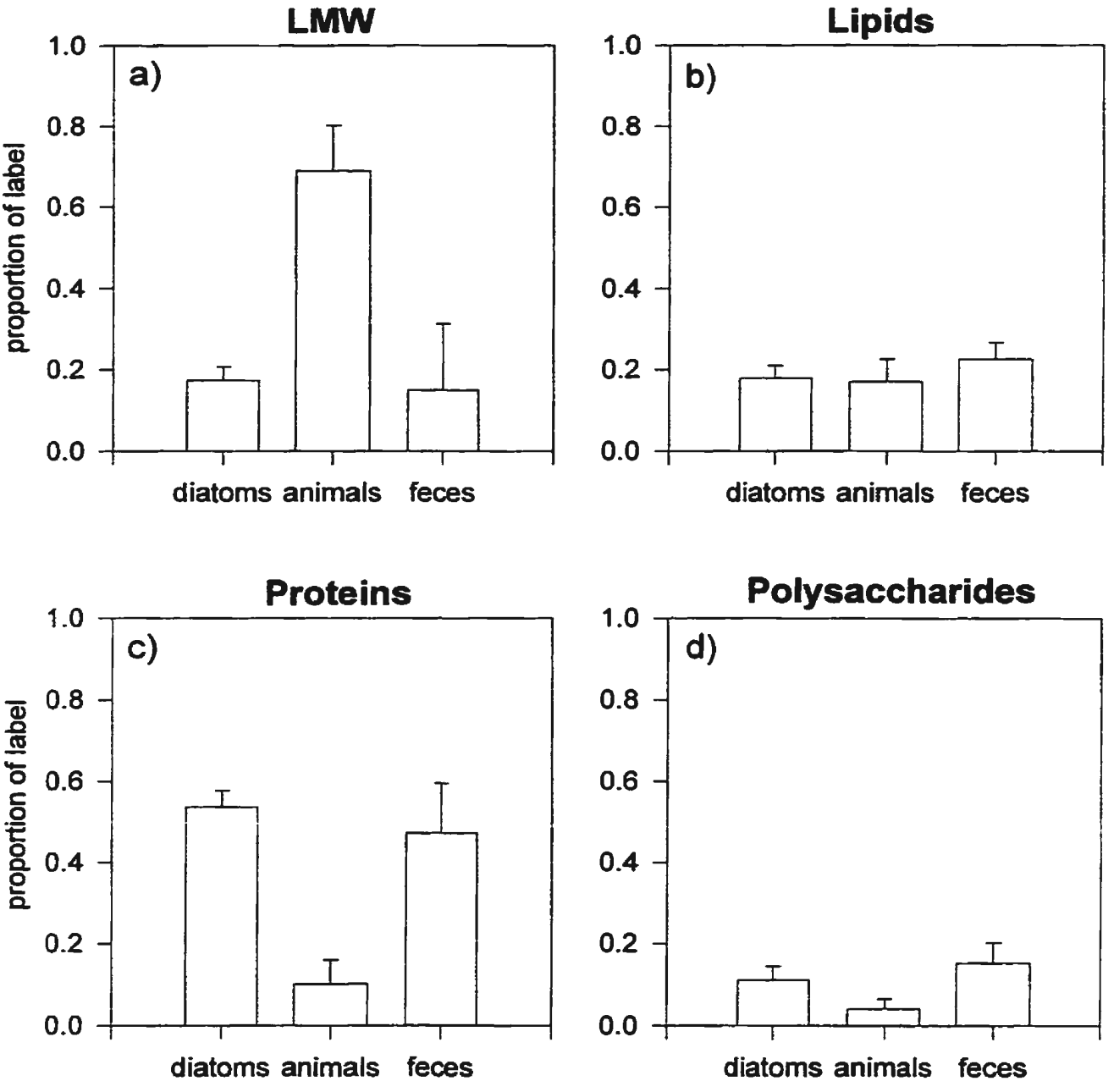
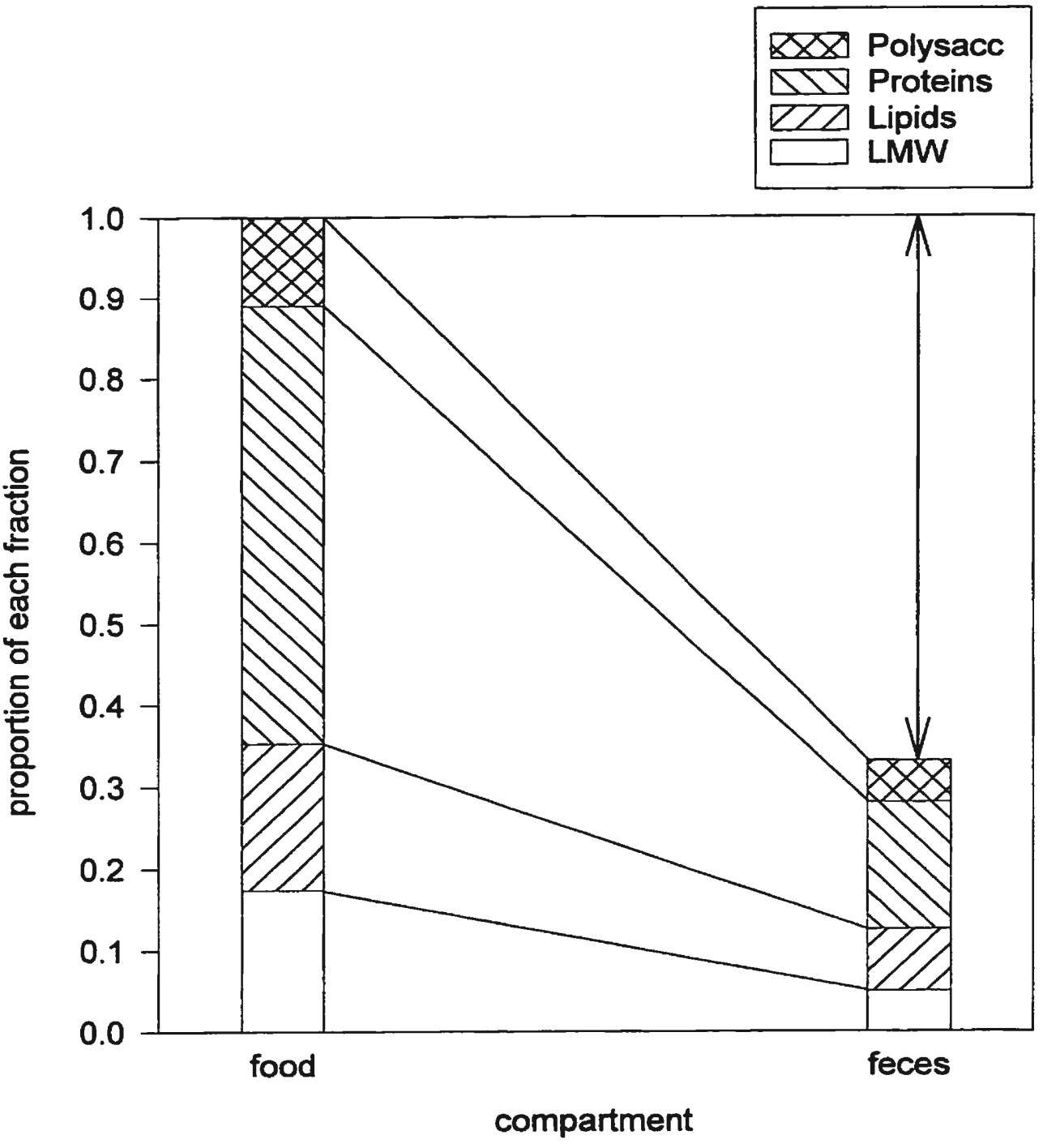

LMW
a)
Lipids
b)
Proteins
c)
Polysaccharides
d)
proportion of label
1.0
0.8
0.6
0.4
0.2
0.0
diatoms
animals
feces

Fig. 4.4. Rationale for calculating assimilation efficiencies for each biochemical fraction knowing their proportion in food and feces and the assimilation efficiency of bulk carbon ($AE_T$).


Polysacc
Proteins
Lipids
LMW
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
proportion of each fraction
food
feces
compartment

# Summary

*Oikopleura vanhoeffeni* was a reliable experimental animal during the 5 years of this study in the sense that it was very abundant during all five field seasons (i.e. from January to June) from 1993 to 1997 and could be collected in surface waters by SCUBA divers. At Logy Bay, the best time for sampling was usually during strong SW winds when small-scale upwelling currents brought colder water containing *O. vanhoeffeni* to the surface. Based upon my personal observations, the highest density of animals occurred in Logy Bay on 12 March 1996, when animals with golf ball-sized houses were probably not more than 30 - 40 cm apart. These pulses of extremely high biomass, the regular presence of the animals during the remaining field season, and the high clearance rates of *O. vanhoeffeni* demonstrate the importance of this species in the Labrador current off Newfoundland. *O. vanhoeffeni* was also a good model organism for laboratory studies. Its large size was advantageous when experimenting with individual animals, and its behavior could be studied without the use of a microscope. Individual animals gave a strong signal in measurements of clearance rate, gut pigments and radioactive counts which allowed collection of data on individual variability. Other zooplankton, such as the much smaller copepods, usually have to be pooled to obtain the same degree of precision. Unfortunately, *O. vanhoeffeni* cannot be kept in culture, unlike the smaller *O. dioica*. Experiments with *O. vanhoeffeni* can be performed for not much longer than three days,

after which the number of animals remaining for experiments is usually less than 10% of the numbers obtained at collection. The reason for the high mortality rate is that the animals frequently collide with the container walls and damage the oikoblast (a fragile array of glands on the trunk which secretes the house). In future, this problem may be alleviated by scaling the size of the container to the animal size, thereby decreasing the probability of collisions with the walls. However, for this particular study it was not a great disadvantage to work with freshly collected animals, since the animals were adjusted to various field situations. In particular, for gut pigments (chapter 3) and assimilation (chapter 4) it was important to work with animals acclimated to field conditions, since I was interested in the digestibility of pigments and carbon. Collections at various times during the field season allowed me to work with a variety of presumably genetically diverse animals with various feeding histories, which gives the data produced in this study a high degree of generality.

The focus of this study was the response of the animals to varying food concentrations while keeping other factors as constant as possible. The response variables examined were clearance rates, as determined by static and flow through experiments (chapter 1), pumping behavior in the laboratory and the field (chapter 1 and 2, respectively), defecation intervals (chapter 1), gut passage times (chapter 3), pigment degradation (chapter 3) and assimilation efficiencies (chapter 4). In the field component (chapter 2), the effect of food concentration was also assessed, although in this particular case neither the range of particle concentrations nor the sample size was sufficient to give

conclusive answers regarding the effect of food quantity on feeding rates. Laboratory experiments, on the other hand, allowed for a highly controlled environment in which ideally only the variable of interest varied. It was therefore surprising to obtain a variety of responses when examining the functional feeding responses on individual animals. Only a relatively small portion of the variance in the feeding activities of the animals (behavior, clearance and ingestion rate) could be explained by food concentration and incubation time. This is likely due to an unidentified variable which was neither controlled for nor measured in the experiments, and which could therefore not be included in the model. I suggest that this unknown factor is the 'quality' of the house. As I showed in the particle removal experiments (chapter 1), the clearance rates were indeed sensitive to the age of the house and the recent feeding history at high particle concentration, indicating interactions of particles with the filter surface. However, even in some relatively new (ca. 1 h) houses, clearance rates were considerably lower than those predicted by the Morris and Deibel (1993) model. Considering that the house is a complicated secretion and does not consist of self-organizing cells, qualities of newly inflated houses even within one individual may be quite variable. Some chambers may not be completely inflated, the food concentrating filter may be perforated or the trunk of the animals may be held in place loosely, thus reducing the amount of water that could potentially be filtered. I have made these observations at various stages throughout my work, but it is very difficult to quantify the quality of the house in these terms. However, considering all the underlying variability, it is surprising how well calculated clearance rates based on two behavioral variables (proportion of time spent feeding and tail beat

frequency) agree with clearance rates measured in the laboratory (chapter 1). This convergence, particularly at particle concentrations of < 100 μg C $l^{-1}$, suggests that it may be possible to use *in situ* behavior in combination with the model to estimate clearance rate in the field. The purpose of this exercise (chapter 2) was to compare entirely different techniques, i.e. lab versus field studies, particle removal versus gut pigment technique, visual observation versus radiotracer experiments. The various estimates for a predetermined size class of animals were not only within a threefold range but also showed a predictable pattern. The behavioral observations gave the highest clearance rate, in other words a physiological maximum performance, whereas the gut pigment technique gave the lowest clearance rates. This was expected since the latter technique measured ingestion rates only, and did not account for particles which remain permanently trapped in the house. At the population level, even a threefold difference in clearance rate seems trivial compared to uncertainties about temporal and spatial distributions of this species.

Many detailed studies on the feeding physiology of animals increase the complexity of models to predict rate processes accurately. However, in this study some examples of constancy were apparent which should be stressed. Clearance rates barely change with food concentration over the range that is representative for most of the year (except the spring diatom bloom, chapter 1). The gut passage times do not vary with food availability (chapter 2), a fact that is at least partly responsible for the independence of assimilation efficiencies and ingestion rates (chapter 4). Assimilation efficiencies for carbon in *O. vanhoeffeni* feeding on diatoms are very close to those reported for

copepods, which means that the use of a common value for various herbivores could be justified in ecosystem models (chapter 4). A mean assimilation efficiency of ca. 70 % is also remarkable considering that *O. vanhoeffeni* is jawless and does not grind the ingested food, and that experiments were performed at a water temperature of 0°C. Biochemical fractions were assimilated to various degrees, but the difference in uptake was insufficient to change the predicted C:N ratios by more than 1.0 (chapter 4). This may have some significance for reducing the complexity of element-specific models of particle fluxes where *O. vanhoeffeni* is a major grazer. Finally, there was only a ca. 10% difference between chl *a* conversion and assimilation efficiencies, which indicates that chl *a* is representative of the labile fraction of carbon in the food. However, care must be taken when comparing these two results, since two entirely different processes were measured. Chl *a* was measured in the animals, whereas carbon was measured in the feces.

A variety of aspects have not been considered in this study, and thus several important questions remain. A central question which was raised in all four chapters was the number of particles which are filtered from the water but do not reach the animal's mouth. To resolve this problem poses a variety of technical and conceptual challenges (chapter 2). When an appendicularian filters for extended periods of time, one can assume that it establishes a dynamic equilibrium between particles outside the house, those currently in the filter and those currently ingested by the animal. However, as the house ages, an increasingly higher percentage of food particles adheres to the fibers, thus giving the food concentrating filter of old houses its conspicuous opaque appearance. When the

animal leaves the house, a portion of the trapped particles in the house is released into the water. Depending on whether the concern is a comparison between ingestion and clearance rates, or contribution of trapped particles in the houses to overall particle flux, different values would apply. To complicate this issue further, particles of various sizes and quality may stick in varying degrees to the fibers of the food concentrating filter. Furthermore, inlet filters are susceptible to clogging by large particles which never enter the house. The percentage of particles trapped in the house in comparison with the amount ingested may also depend on the ambient food concentration (Acuña, pers. comm.). Most of the animals in this study derived from a size range from ca. 2 to 5 mm. In the functional response chapter (1), body sizes were purposely narrowed to avoid potential allometric effects. Since this range did not cover the entire range of juveniles of *O. vanhoeffeni*, it may be very interesting to investigate the influence of body size on the functional feeding response and other variables in more detail. Finally, another important aspect of the feeding ecology of *O. vanhoeffeni* which has not been studied is the investigation of food sources other than diatoms. *O. vanhoeffeni* filters large amounts of colloidal material, bacteria, flagellates and ciliates, and data on feeding and digestion may vary according to the type of particle examined. However, I hope that my work with *O. vanhoeffeni* has closed a few of the gaps in our knowledge of appendicularian feeding, and that it will inspire some new ideas for future research on these interesting and beautiful animals.

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